Ciprofloxacin-Gold Nanoparticles Loaded Polymeric Membrane as a Novel Antibacterial Wound Dressing against Diabetic Foot Ulcer Infection

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A thesis submitted in partial fulfillment of the requirements for the degree of

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Thesis Supervisor:

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Dedicated to my parents.

Mr. and Mrs. Malik Bashir Ahmad Kamboh

for their endless love, support, and encouragement

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Abstract

Nano-based wound dressings, with improved features such as controlled release of therapeutic drug and increased antibacterial activity, play a significant role in supporting efficient and fast wound healing. This research describes a unique method for creating a polymeric wound dressing material to address the problems of diabetic foot ulcers (DFUs) infection. The research focuses on introducing ciprofloxacin-loaded gold nanoparticles (CIP-AuNPs) into the dressing material using gum tragacanth (GT) and polyvinyl alcohol (PVA). Ultraviolet-Visible spectrophotometry (UV), Fourier transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM), Energy Dispersive X-ray Spectroscopy **(**EDX), Contact Angle Measurement, and Mechanical Test were used to characterize the fabricated membranes Biocompatibility, Drug Release Efficiency, and Antibacterial Efficacy against *E. coli* were all investigated. In Vitro results showed the effective synthesis of CIP-AuNPs as well as the improved mechanical characteristics of the membranes. The CIP-loaded membrane demonstrated low cytotoxicity and prolonged ciprofloxacin release. The antibacterial activity of the CIP-loaded membrane was substantial. In vivo testing on rat model showed the valuable and efficient results of CIP-loaded membrane on wound healing and potential for the clinical trials. This research suggests a viable wound dressing material for the treatment of DFUs.

Keywords: *Diabatic Foot Ulcer, PVA, Cip-AuNPs, Gum T., Biocompatibility*

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Chapter 1 Introduction

1.1 Introduction

Diabetes Mellitus is a chronic disease that causes high blood glucose. This occurs when the body is unable to produce insulin, resulting in hyperglycemia, or when the body is unable to utilize insulin efficiently. In 2019, it was predicted that roughly 463 million individuals globally were affected by this condition, with this figure expected to rise to 700 million by 2045 [1]. Diabetes' consequences include cardiovascular disease, blindness, renal failure, and foot ulcers [2].

Diabetic Foot Ulcer is a frequent diabetic complication among patients with uncontrolled diabetes (REF). Foot ulcer wounds take longer to heal and involve hospitalization and amputation in many cases. It has been reported that one leg gets amputated every 30 seconds from diabetic wounds, which results in 50-70% of limb amputations [3]. Deformity, microcirculation dysfunction, peripheral neuropathy, peripheral arterial disease, kidney disease, macro-vascular disease, cardiovascular events, and growth factor (GF) activity and expression delay diabetic wound healing [4].

Chronic wound dressings need specific features. Ideal polymeric dressings have gaseous permeation, high porosity, excellent antimicrobial activity, mechanical properties, and the ability to deliver bioactive agent [5].

Nanobiotechnology advancements have contributed to the development of wound-healing materials that are both cost-effective and efficient. There are several nano-drug delivery strategies for skin tissue regeneration that have been discovered and employed [6,7]. Metal nanoparticles can repair wounds as compounds and molecules. ZnO kills bacteria and fungi by collecting reactive oxygen species on their surfaces and promoting ROS production [8]. Zn, being the major component of metalloproteinase, can help to build extracellular matrix and aid wound healing [9]. In addition, both gold nanoparticles (AuNPs) and silver nanoparticles (AgNPs) have been shown to be useful in the treatment of a wide range of medical diseases, such as infections caused by bacteria, viruses, and fungi [10]. In vivo wound healing was evaluated in rats to AuNPs and AgNPs monotherapy; AuNPs treatment revealed considerable free radical scavenging action as well as enhanced wound healing [11].

Many antibacterial drugs have been used to prevent bacterial infection of the incision for wound healing because bacterial infection of the incision must be avoided for wound repair. One of the most well-known antibiotics is Ciprofloxacin (CIP). It can be used to treat a variety of wound infections since it has antibacterial activities against gram-positive and negative bacteria [12].

Gum Tragacanth is a well-known natural gum that possesses several desirable qualities, including biodegradability, non-toxicity, biocompatibility, and availability. Because of these qualities, it is excellent for applications involving drug carriers and the regeneration of skin. The manufacture of pure GT polymeric wound dressing on the other hand is limited because of their unique chemical structure, and it is required that it can be combined with biodegradable synthetic polymers such as polyvinyl alcohol (PVA) [13,14]. Moreover, to improve mechanical performance, natural-based polymeric wound dressings are often cross-linked with synthetic polymers [15].

Polyvinyl Alcohol, a chemically stable polyhydroxy polymer has good adhesive qualities. and often used as a cross-linker [16]. PVA is non-toxic, water-soluble, and biodegradable, with excellent heat and flexibility. However, due to its low flexibility, hard membrane, and highly restricted hydrophilic characteristics, using only PVA as a wound dressing material has significant disadvantages. These limits can be circumvented by crosslinking and combining PVA with other biopolymers [17].

The significance of this thesis is based on the development of an innovative wound dressing material that has the potential to address the limitations observed in existing therapies for diabetic foot ulcers. The objective of this study is to investigate the potential benefits of the PVA/GT membrane in terms of enhanced antibacterial properties, increased mechanical strength, improved biocompatibility, and enhanced moisture retention. This will be achieved by incorporating gold nanoparticles and the wide-range antibiotic ciprofloxacin into the membrane. The anticipated outcome of this research is to facilitate improved and faster wound healing and reduce infection rates specifically in diabetic foot ulcers. The investigation into wound care for individuals with diabetes will be enhanced by the discoveries derived from conducting in-vivo experiments on a rat model. These experiments will provide valuable insights into the efficacy of the substance under study and its potential for practical implementation in a clinical aspect.

1.2 Objectives

- To Synthesize and Characterize CIP loaded AuNPs.
- To Synthesize and Characterize PVA/GT Membrane with CIP loaded AuNPs.
- To Evaluate the Effect of CIP-AuNPs on Antibacterial Activity, Mechanical Properties, Biocompatibility and Wettability of PVA/GT Membrane.
- To Conduct In-Vivo Testing of Membranes on Rat Model of Diabatic Foot Ulcer Infection.

Chapter 2 Literature Review

2.1 Diabetes Mellitus

Diabetes mellitus is a metabolic disorder that is characterized by hyperglycemia and glucose intolerance. It has been associated with reduced insulin secretion, decreased sensitivity in the periphery, and malfunction in B- cells. Diabetes is one of the oldest diseases in the world [18]. According to the International Diabetes Federation, there were 451 million adults around the world who were diagnosed with diabetes in 2017. It is projected that 693 million people would have diabetes by the year 2045. According to figures estimated by the World Health Organization (WHO), diabetes would affect more than 19% of the adult population of the world by the year 2030 [19]. Diabetes has been a common cause for concern due to the high rates at which it both causes new cases and results in death, as well as the significant costs associated with both its management and treatment. Diseases related to diabetes are more prevalent in developing nations, as more than half of the total number of cases remain undiagnosed.

2.1.1 Classification and Pathophysiology of Diabetes Mellitus

Diabetes Mellitus is divided into four types:

- Diabetes Mellitus Type 1
- Diabetes Mellitus Type 2
- Gestational Diabetes Mellitus
- Secondary Diabetes Mellitus

Type 1 DM manifests clinically as hyperglycemia due to acute or chronic insulin deficiency in the blood. In **type 2 DM**, the pancreatic islets of Langerhans are hypersensitive to glucose in plasma, resulting in the secretion of higher-than-normal insulin levels in the systemic circulation. Hyperinsulinemia is evidence of an attempt to offset hyperglycemia, which further deteriorates and affects B-cell function [20,21]. **Gestational DM** is a type of glucose intolerance in pregnant women, while **secondary DM** is caused by hereditary issues in beta-cell function or insulin action, pancreatic diseases, or medications or chemicals [22].

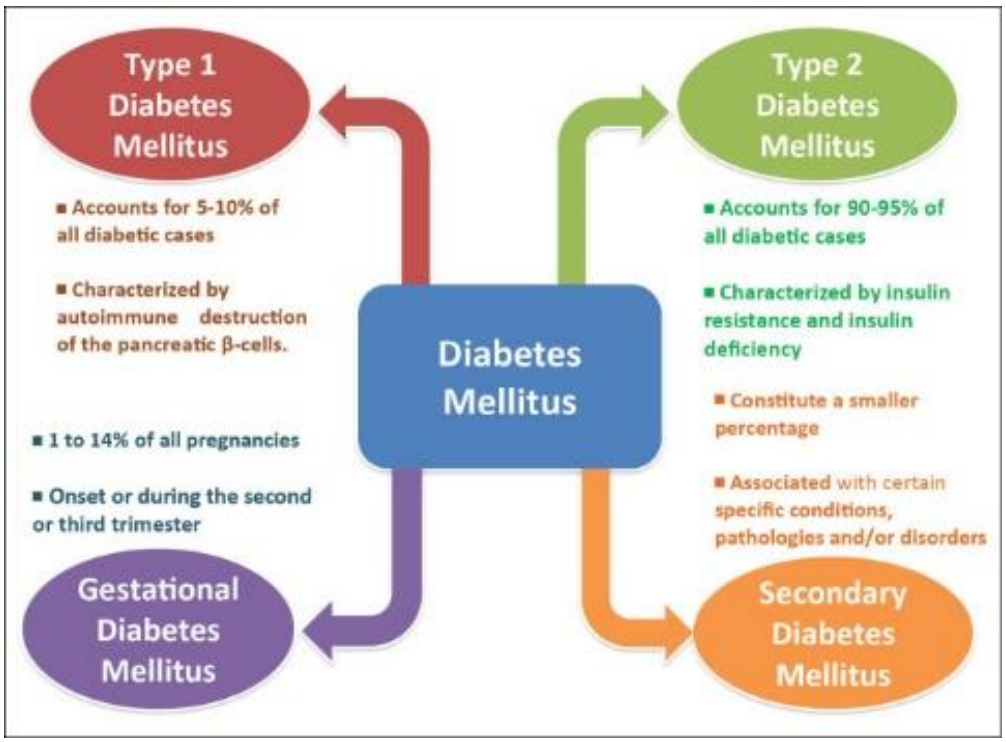

Figure 1 Types of Diabetic Mellitus [23].

2.2 Complications of Diabetes Mellitus

The metabolic processes of glucose, lipids, proteins, and electrolytes are all impacted by diabetic complications produced by hyperglycemia (HG), and all these changes have the potential to cause damage to the vascular system. Under these circumstances, a substantial number of endothelial capillary cells, which are found in the retina, glomerular, and nervous system are killed off because of an excessive accumulation of toxic glucose [24]. Chronic hyperglycemia (HG), decreased lipid catabolism, increased ROS production, and a weakened antioxidant protection system are the primary causes of the essential mechanisms involved in the development of diabetes complications. These factors all contribute to insulin resistance and increased damage to pancreatic beta-cells [18].

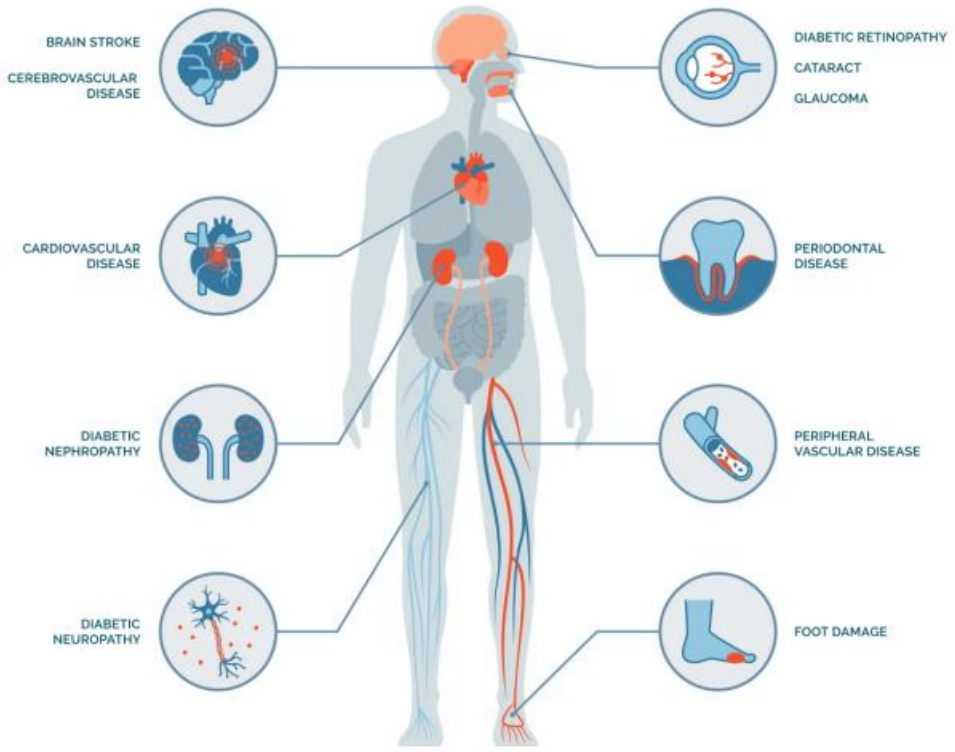

Figure 2 Complications of Diabetic Mellitus [25].

2.2.1 Vascular Complications

There are two types of vascular problems that can happen with diabetes: macrovascular and microvascular. Macrovascular problems include coronary and peripheral arterial disease, while microvascular problems are linked to poor vascular permeability and affect organs and tissues like the kidneys, retina, and nerves [26].

2.2.1.1 Macrovascular Complications

Many reasons contribute to endothelial cell dysfunction in macrovascular problems, including high blood glucose levels and inflammatory issues [27]. Diabetes is also related with an increase in ROS generation, which can cause inflammatory issues, vasoconstriction, and accelerated lipid peroxidation, which contribute to atherosclerosis, a condition characterized by excessive lipid deposition, particularly of low-density lipoprotein in the subendothelial layer of

major blood arteries. It is more common in diabetic people than in non-diabetics. Furthermore, atherosclerosis increases blood vessel endothelial penetrability, which is prevalent in diabetes with more inflammation, too much ROS is released, which speeds up the oxidation of phospholipids and sterols. This makes the antioxidant and anti-inflammatory benefits of high-density lipoprotein (HDL) less effective. Other macrovascular complications, such as calcification and plaque formation, can lead to the development of severe vascular complications such as coronary artery disease (CAD) and stroke which are common in individuals with diabetes and have a high death rate [28].

2.2.1.2 Microvascular Complications

One of the microvascular complications is **diabetic nephropathy** a condition that can be both inherited and caused by specific environmental factors is linked to structural changes in the kidney's endothelial cell barrier and the basement membrane that increase the filtration of protein in urine, indicating that protein breakdown is disrupted in the diabetic patient [29]. One-third of patients with uncontrolled diabetes may develop diabetic nephropathy, requiring renal dialysis [30]. In addition, **diabetic retinopathy** is another complication in which in several cases, diabetes is diagnosed indirectly through an eye exam for impaired vision. Without treatment, diabetes can lead to blindness. The potential risk of blindness in diabetics is associated with a long-term development of retinopathy, which has been shown in most patients for decades, with the risk of losing eyesight increasing over time [31].

2.2.2 Diabetes Neuropathy

After years of HG, the onset of diabetic neuropathic problems is accelerated chronic HG can cause nervous system dysfunctionality such as arrhythmias, sexual dysfunction, and gastroparesis. Long-term diabetic patients, on the other hand, may develop one or more forms of neuropathies. One is **peripheral neurological disorders** that induce sensory or sensorimotor neuropathy increase the chance of foot ulceration and amputation [32]. The other is **autonomic neuropathy**, which includes diabetics and gastrointestinal dysfunctionality, may result in aberrant digestive system function. Diabetics with autonomic neuropathy may experience nausea, vomiting, abdominal distress, and heartburn [33].

2.3 Diabetic Foot Ulcer

Diabetic foot ulcer (DFU) is one of the most common, dangerous, complicated, and expensive diabetes consequences. Diabetic foot ulceration is caused primarily by the coexistence of neuropathy and ischemia. Diabetes is linked to an increased risk of foot injuries owing to reduced proprioception. Ischemia causes poor healing in the wounded areas, and superimposed contaminations cause ulceration. Diabetes is related to morbidity due to limb amputation [34]. Even with adequate medical care, persistently unsuccessful wound healing in diabetic foot injury might occur. Foot amputation is justified in cases of chronic, non-healing foot ulcers. In diabetes, all phases of complicated wound-healing cascade are compromised, which is exacerbated by a variety of variables such as inflammation, proliferation, lead to failure of wound-healing [35].
Diabetic feet have become more common as the global prevalence of diabetes and diabetic patients' life expectancy has increased. Foot ulcers are estimated to occur annually in 9.1-26.1 million diabetics worldwide. Diabetes will affect between 19 and 34% of persons at some time in their life [36].

2.3.1 Pathophysiology of Diabetic Foot Ulcer

The pathogenesis of diabetic foot ulcers is explained by a combination of arterial occlusive disease, peripheral neuropathy, trauma with sequential infection, and arterial occlusive disease. Peripheral neuropathy results in intrinsic muscle atrophy, which leads to hammer-toe formation and the formation of elevated pressure zones on the plantar area of the foot near the metatarsals. Walking-related repetitive stress, in conjunction with decreased sensitivity, predisposes to skin damage by causing atrophy and displacement of protective plantar fat pads, leading to infection. Neglecting skin care, such as failure to apply moisturizing lotions or failure to recognize cutaneous stress such as redness and blister formation can lead to injury which can cause tissue damage if not addressed timely [37]. **Figure 3** shows the wound healing process in normal and diabetic person.

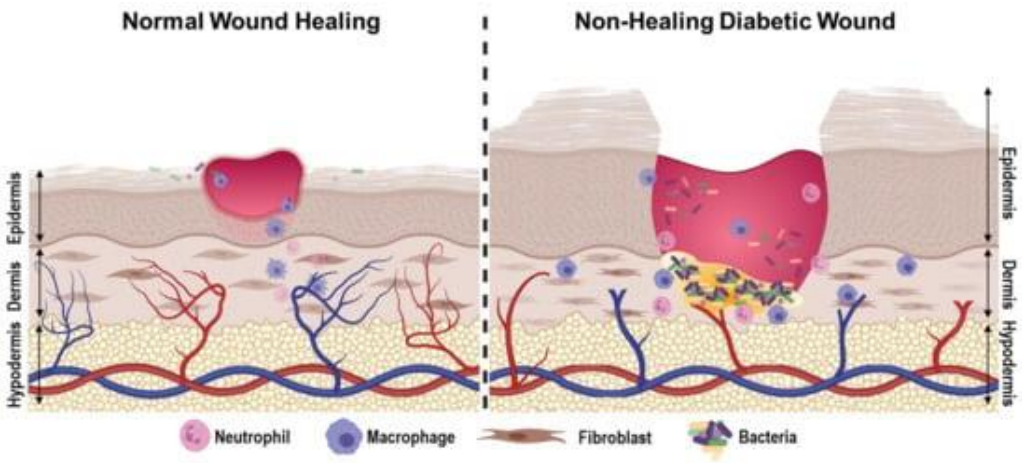

Figure 3 Normal Wound Healing (left) Non-Healing Diabetic Wound (right) [38].

2.3.2 Management of Diabetic Foot Ulcer

Developing an effective treatment strategy for a persistent, resistant lesion remains a challenge in DFU management. **Table 1** shows the traditional types of treatments that are available for the management of DFU. Each treatment has its own pros and cons.

Type of treatment	**Advantages**	**Disadvantages**
Glycemic Control	It has reduced the risk of infection and improved wound healing.	It has hypoglycemia risk and has individual variability.
Pharmacological Therapy	It Reduce the risk of infection and control inflammation.	It has adverse effects and risk of medication interactions moreover cost is also a drawback of it.
Wound Dressing	Moisture management, non-adherent, gentle removal, and patient comfort are the advantages of it.	Some of the dressings are costly and difficult to use.

Table 1 Pros and Cons of DFU Treatments

However, a greater understanding of the molecular biology and the pathophysiology of long-term wounds should lead to a more effective and preferable treatment paradigm for chronic wound care. Researchers believed that standard guidelines were sufficient for promoting wound healing [39]. such as:

- Dressing the wounded area with adequate biomaterials that can prevent infection of the affected area over the long-term duration of wound management providing an ideal moist environment to promote wound healing.
- The use of a medicated dressing capable of providing prolonged and efficient release of manufactured pharmacological moieties and biomolecules

2.3.3 Available Wound Dressings

The primary functions of conventional wound dressings are to prevent further damage from the environment, to stop bleeding, to provide comfort, and to absorb wound exudate. Examples include wool dressings, plaster, gauze, and bandages [40].

Composites, sprays, foams, films, and gels are all examples of interactive dressings that can promote re-epithelialization and granulation, keep the wound moist, and speed up the healing process. These dressings can also include bioactive ingredients [41] .Wound dressings made of **foam or wafers** combine hydrophobic and hydrophilic foam with bio adhesive boundaries to create a solid, porous wound dressing **(Figure4)**.

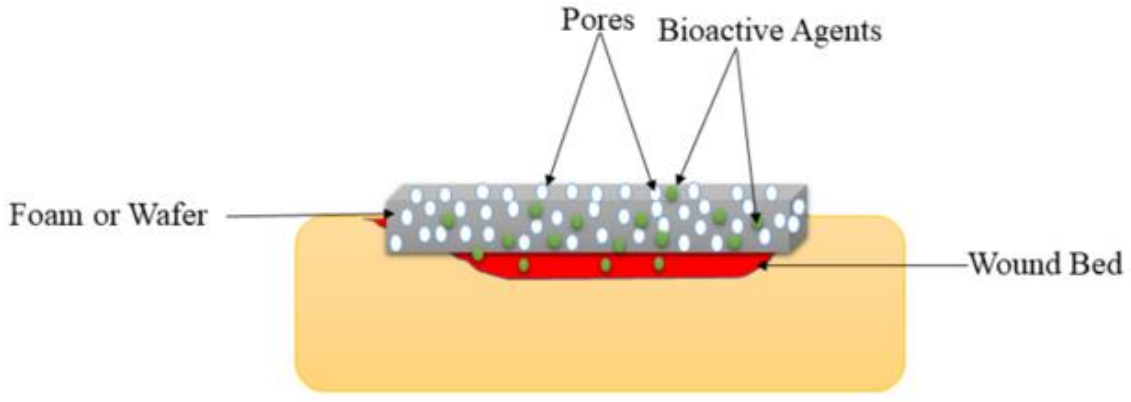

Figure 4 Foams for Wound Healing [42].

Films are a type of wound dressing material that sticks to the wound and are translucent, allowing gases like carbon dioxide, oxygen, and water vapor, to infiltrate between the lesion and the environment. These dressings aid in the body's natural process of removing dead tissue from the injury site. There is no need for additional tapping because polymer-based films offer excellent mechanical qualities such as high elasticity and flexibility [43].

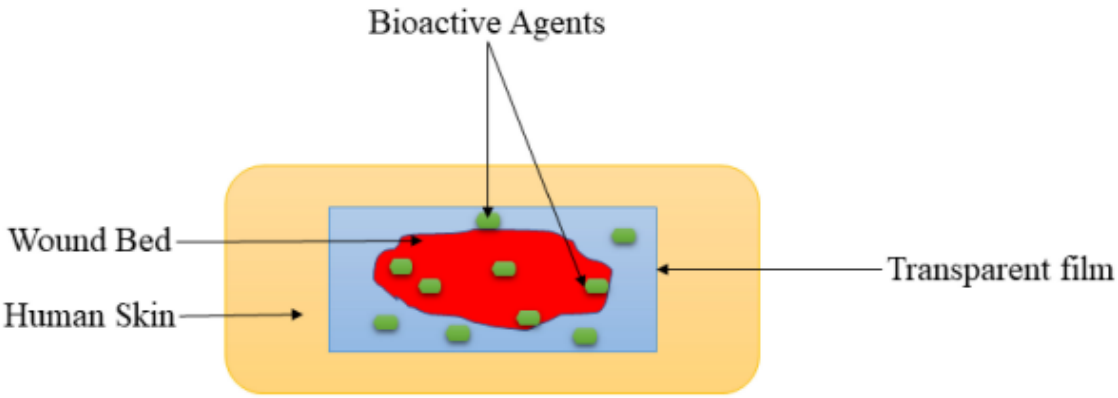

Figure 5 Films for Wound Healing [42].

In the fields of dermatology and plastic surgery, dermal grafts are among the most crucial materials. Skin grafts can be either allografts, autografts, or xenografts, all of which are acellular. Traumatic wounds, burn reconstruction, scar contracture hair restoration, and congenital skin insufficiency are all treated with these products [44].

Hydrogels, which are networks of cross-linked polymers in three dimensions, include humidity levels of 90% or more. They are ideal for use as wound dressings due to their adhesive properties, adaptability, and similarity to biological tissues. Healing is accelerated, pain is relieved, infections are prevented, and the surrounding area is kept moist to facilitate cell migration. Hydrogels lessen the harmful effects of drugs and other treatments [45,46].

2.3.4 Limitations of Available Wound Dressings

Some traditional dressings have defects, such as wound exudate leakage, which can lead to bacterial infections and skin injury during removal. Some interactive dressings, such as foams, can improve exchange of gases, protect against maceration, provide adequate hydration for rapid healing, and absorb enormous volumes of exudate. However, they are ineffectual on dry wounds,

injuries with inadequate exudation, and dry scars. The patient's comfort can be assured by setting parameters correctly. can cause wound infections, transmit disease, are rejected by the body, are costly, and have a limited life. There is a concentration issue in hydrogels. S. Lai et al. investigated the use of plant extracts for diabetic ulcer lesion treatment. Hydrogels improved wound healing, re-epithelialization, and closure in rodents. On the other hand, extract-loaded hydrogels with a high concentration (4%) may promote inflammation and increase wound size, whereas extract-loaded hydrogels with a concentration of 2% did not [47]. In contrast, derma dressings are inappropriate for the treatment of complex injuries. **Table 2** shows the pros and cons of various polymers that can be used for wound healing.

Polymers	Indication	Advantages	Disadvantages
Hydrogel	Efficient for wounds with minimal or no exudates	Non-adherent and can be applied or removed without seriously interfering. with the wound bed.	Not efficient in wounds with excessive exudates which may require secondary dressings, not too absorptive
Foam	Efficient for greatly exudative wounds especially when drainage is at peak.	Comfortable and conformable, easy application and removal without interfering with wound bed	Opaque and thus does not allow the visualization of wound bed and restricted to excessive exudative wounds only
Hydrocolloid	Efficiently manage wounds with mild-to-moderate draining exudates	Conformable for easy application and reduce pain, inflammation. and progression at the wound site.	Degradation of the dressing materials may produce a residue of varying colors and foul odor.
Film	Efficient for wounds with mild-to-moderate exudates.	Waterproof and gas permeable. Maintain a moist wound environment which helps to clear erotic. debris and cleanse the wound bed.	May have potential risks of damaging wound bed while removing film dressing because of its adhesive nature, non-absorptive and will be overwhelmed. by moderately exudative wounds.

Table 2 Pros and Cons of several types of polymers [15].

2.4 Nanotechnology in Wound Healing

Nanotechnology has emerged as a method to wound healing, with different nanoscale techniques being investigated for distinct stages of restoration. Nanomaterials are employed in wound healing in two ways: as inherent features that enhance therapy and as therapeutic agent delivery vehicles. Many advancements have resulted in the development of cost-effective and

efficient materials, such as nano-drug delivery systems for skin tissue regeneration. Nanomaterials are increasingly being employed in wound healing, with two categories widely used: intrinsic qualities that enhance treatment and therapeutic compounds as delivery vehicles [6].

2.4.1 Metal Oxide Nanoparticles

2.4.1.1 Silver Nanoparticles (AgNPs)

The antibacterial property of Ag in nature has been known for quite some time, and AgNPs were among the first nanoparticles used in skin healing. Silver has no antibacterial properties in its solid state and only biological effects when dissolved in water. Silver can inhibit the function of the cytochrome in the bacterial respiratory chain by interacting with it. Silver also promotes wound healing by regulating cytokine levels in the lesion, reducing inflammation and lymphocyte infiltration, and promoting epithelial reconstruction [48]. In addition to their inherent antibacterial effect, AgNPs can enhance silver's antibacterial potential by interacting with other substances or produce antibacterial effects in other ways. Zhou et al. discovered that the Ag/AgCl/rGO nanoparticles possess bactericidal properties by promoting the production of oxidation free radicals [49].

2.4.1.2 Copper Nanoparticles (CuNPs)

Copper nanoparticles release Cu2+, which alters the function of the enzyme by hardening its protein structure, allowing it to destroy bacteria. Copper has a significant inhibitory effect on a variety of fungi and drug-resistant bacteria, which is exceedingly beneficial for diabetic chronic wounds, as demonstrated by laboratory tests [50,51].

2.4.1.3 Zinc Oxide Nanoparticles (ZnO NPs)

The antimicrobial activities of zinc oxide (ZnO) nanoparticles have been improved, rendering them effective against therapeutically significant microbial pathogens such as *Staphylococcus aureus, Pseudomonas aeruginosa, and Escherichia coli*. Recent in vitro and in vivo research has focused on these nanoparticles for the treatment of wound infections [52]. Recent research has demonstrated that Zn2+ ion release from ZnO NPs is a key mechanism for oligodynamic actions against eukaryotic and prokaryotic cells.

2.4.2 Limitations of Metal Oxide Nanoparticles

Even though it has been demonstrated that silver (Ag) possesses antibacterial, antifungal, and antiviral characteristics, the direct administration of silver to the wound site might result in several complications. Silver does have some level of toxicity to living organisms, and silver by itself at the wound site is unable to maintain a stable concentration of silver, which can lead to local aggregation and adverse responses. Additionally, the wound requires a moist and airflow which AgNPs are unable to provide [53].

Copper's antibacterial effect is dependent upon dose, and excessive quantities are toxic to cells; therefore, dressing design should take this into account. The uncontrolled release of Zn2+ ions under certain situations has been recognized as a significant impediment to obtaining an adequate industrial formulation. As a result, a large dosage of NPs is required, with the potential of neutralizing the therapeutic benefits of the formulation, as well as increased side effects and preparation costs.

2.5 Current Study

Membranes, which are utilized as wound dressings, possess a structural composition that closely resembles that of films. Membranes exhibit a diverse array of functions that set them apart from films. Polymeric membranes exhibit the remarkable capability to effectively absorb surplus exudate, thereby facilitating the maintenance of an optimal moist environment that is conducive to the healing of wounds. Furthermore, these membranes possess the ability to retain biological fluids even when subjected to pressure, thereby minimizing the need for frequent dressing changes. Additionally, they contribute to minimizing disturbance to the wound bed and may potentially exhibit cleaning properties. Moreover, membranes exhibit favorable mechanical attributes, including remarkable flexibility, optimal comfort, inherent softness, and notable stretchability [54].

Polymer-based membranes filled with bioactive compounds were suggested to have potential for diabetic wound care treatment by certain studies. Because of their abundance, biocompatibility, and biodegradability, natural polymers offer major advantages over manufactured polymers. Natural polymers can also be easily modified chemically and biochemically. **Gum Tragacanth** has an extremely extended shelf life and is extensively used as

an emulsifier in food, medicines, and related sectors. GT has been discovered to be a beneficial plant-derived chemical in a variety of healthcare-related applications [55]. Because of its excellent stability throughout a wide range of pH and temperature. Plant-derived polymers, such as drug delivery methods, Due to the distinctive chemical composition of pure GT nanofibers, their production is inherently constrained, leading to use with synthetic biodegradable polymers [14].

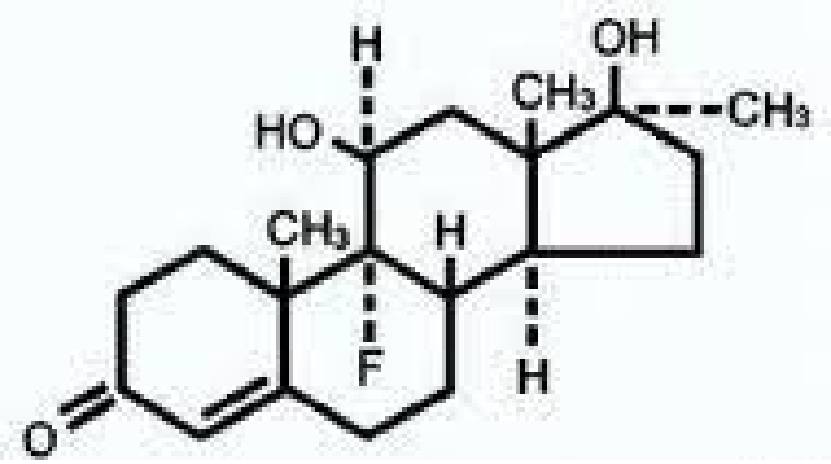

Figure 6 Structural Formula of Gum Tragacanth [56].

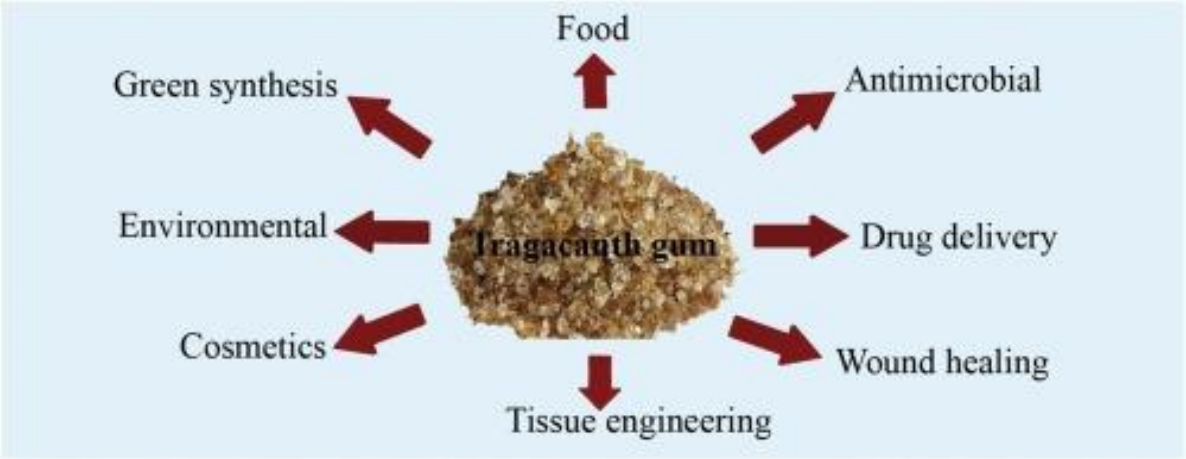

Figure 7 Applications of Gum Tragacanth [56].

Polyvinyl Alcohol (PVA) is a polyhydroxy polymer renowned for its exceptional chemical resistance and remarkable adhesive properties. Polyvinyl alcohol (PVA) is frequently employed as a cross-linker in various applications owing to its remarkable characteristics, including its ability to form membranes and its hydrophilic nature. Polyvinyl alcohol (PVA) is a substance that possesses several desirable characteristics, making it a valuable material for various applications. Firstly, PVA is known to be non-toxic, ensuring its safety for use in different settings. Additionally, it exhibits water solubility, allowing for easy dissolution in aqueous environments. This property is particularly advantageous in applications where PVA needs to be easily removed or dispersed. Moreover, PVA is biodegradable, meaning it can be broken down by natural processes over time, reducing its environmental impact. Lastly, PVA demonstrates favorable heat resistance. Nevertheless, it is important to acknowledge that the utilization of PVA hydrogel as a standalone wound dressing polymeric material is subject to certain limitations. These limitations primarily stem from its inadequate elasticity, rigid membrane structure, and highly constrained hydrophilicity characteristics. The mentioned limitations can be effectively reduced through the implementation of a strategy involving the combination of polyvinyl alcohol (PVA) with various biopolymers via the processes of crosslinking and blending, as suggested. The potential of these interactions lies in their ability to enhance the absorption and mechanical properties of PVA in aqueous solutions and physiological fluids, thereby leading to an improvement in its biological activity [14].

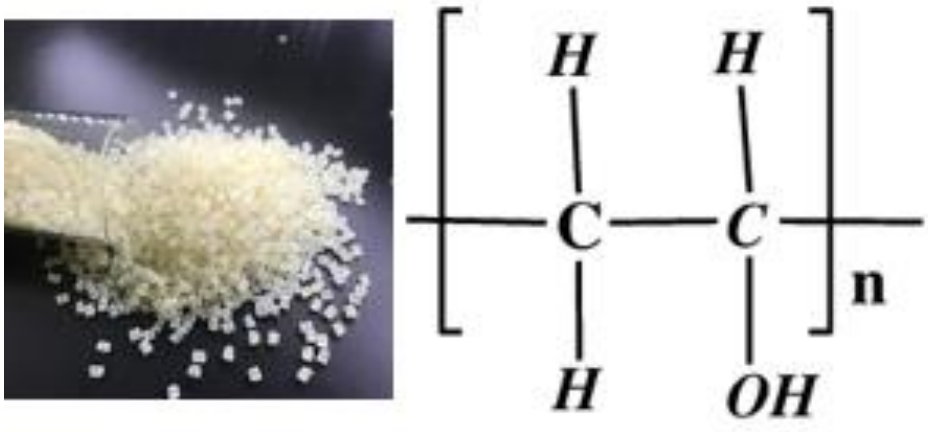

Figure 8 Structural Formula of Polyvinyl Alcohol [57].

By interfering with ATP generation, altering bacterial proliferation, and reducing bacterial growth, **gold nanoparticles (AuNPs)** can cause bacterial metabolic dysfunction. AuNPs also have antioxidant properties, which help to reduce reactive oxygen species and inflammatory cells. AuNPs can strengthen their antibacterial activities when coupled with other antibacterial medicines, making them an attractive contender for combined wound healing applications. In animals, a wound dressing containing dextran/sericin and AuNPs expedited wound healing and decreased scar formation [58].

Parenteral broad-spectrum antibiotics that have demonstrated clinical efficacy in the treatment of diabetic foot infections are highly recommended for cases of severe infection. These antibiotics encompass imipenem/cilastatin, newer fluoroquinolones such as levofloxacin and ciprofloxacin, third or fourth generation cephalosporins like ceftazidime and cefuroxime, as well as betalactam/betalactamase inhibitors. A wide variety of antibacterial agents have been employed for the purpose of mitigating bacterial infection of the surgical incision, among which **ciprofloxacin (CIP)** stands out as a widely recognized antibiotic. The utilization of this therapeutic agent exhibits efficacy in addressing a diverse range of wound infections, owing to its notable antibacterial properties that effectively combat both gram-negative and gram-positive bacterial strains [12].

Chapter 3 Materials and Methods

Sigma Aldrich supplied polyvinyl alcohol (MW = 72,000 Da), 0.5Mm trisodium citrate and chloroauric acid (MERK, Munich, Germany). Gum Tragacanth was purchased from the local market. Deionized water was used throughout the experiment. The rats were taken from Atta-Ur-Rahman School of Applied Bioscience, National University of Science and Technology.

3.1 Synthesis of AuNPs and Cip-AuNPs

The AuNPs were made by using a technique that has been described in the literature [59]. At 100°C, 50 ml of chloroauric acid (0.5 millimolar) were fluxed while 5 ml of Trisodium citrate solution was added drop by drop while being stirred with a magnetic stirrer. Citrate acts as a stabilizer and pH-lowering agent. A red wine hue proved that particles were being formed. CIP was loaded onto AuNPs by first creating a stock solution of CIP at 0.0008 g/ml in a volume of 50 ml. 20ml of the solution (pH 6.5) and 5 ml of CIP (0.5 mM) was mixed. After that the solution was shaken till the red color faded and was replaced with a blue-purple one **(Figure 9)** [60].

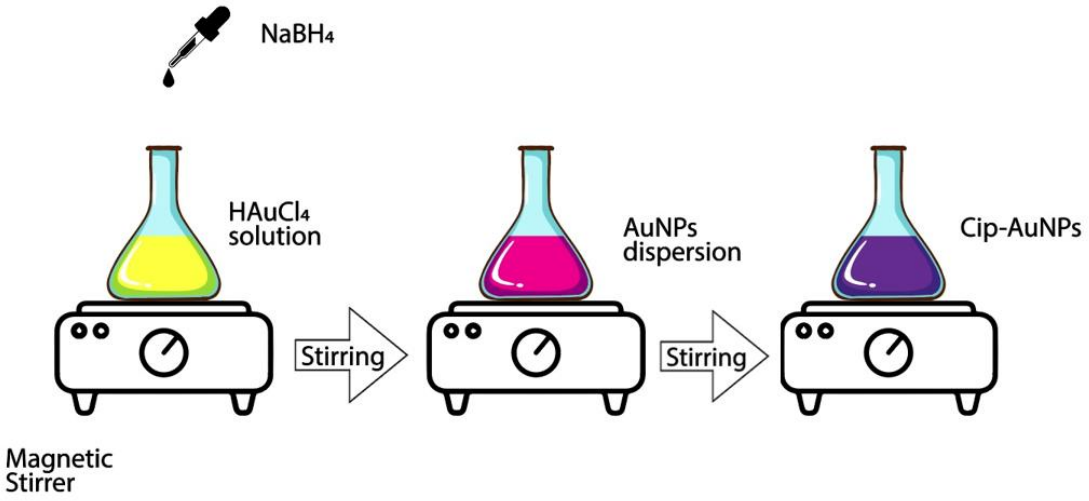

Figure 9 Preparation of CIP-loaded AuNPs

3.2 Synthesis of Membranes

PVA and GT solutions (10 and 0.5 wt. %) in deionized water were prepared **(Figure 10)**. PVA/GT homogenous aqueous solution was made by combining PVA and GT in a 60/40 (w/w) ratio [61]. For membranes preparation, a casting method was used **(Figure 11)**. AuNPs and Cip-AuNPs were put into PVA and GT solutions, and then blended solutions were cast on 8cm diameter petri plates as membranes were to be prepared by casting method and dried overnight. The dried membranes were removed [62].

Figure 10 5 wt.% GT Sol.(left) and 10wt% PVA Sol.(right) in DI Water

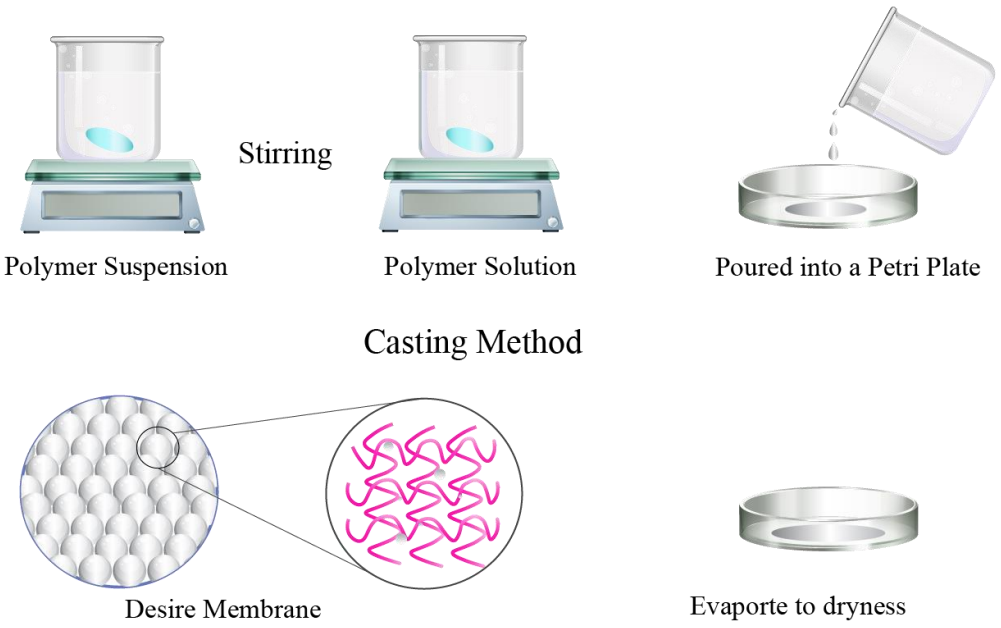

Figure 11 Preparation of Polymeric Membrane by Casting Method

3.3 Characterization of Nanoparticles

Multiple characterization techniques were conducted such as UV-Vis spectrophotometry for the confirmation of synthesis. To assess functional groups FTIR, SEM for surface morphology, EDX for element mapping.

3.3.1 UV-Vis Spectrophotometry

Preparation of nanoparticles was confirmed by using UV-VIS spectrophotometry (UV-2800 BMS Biotechnology Medical Services, Madrid, Spain). In the UV-Vis spectrometer, a cuvette with a sample was put in front of the UV lamp. Different wavelengths of light from the lamp hit the sample, and the absorbance at each wavelength was recorded. The lambda maxima are the largest amount of sample that can be absorbed, which is related to the amount of sample in the cuvette. It is also called molar absorptivity, and it is used to compare the wavelengths of different chemicals.

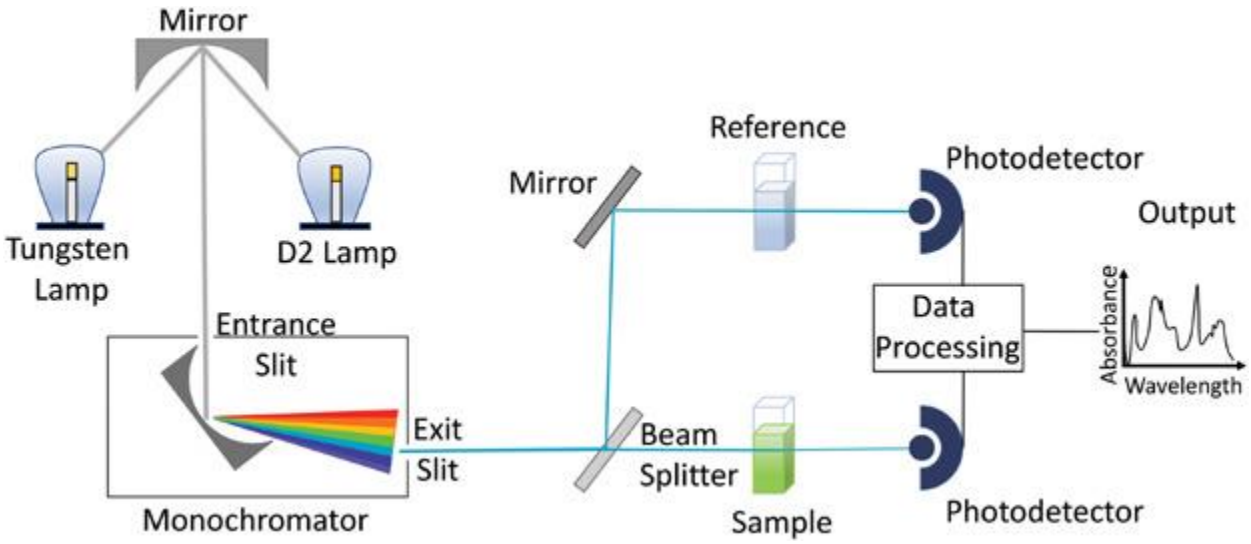

Figure 12 Working Mechanism of UV-Vis Spectrophotometry [63].

3.3.2 FTIR Analysis

FTIR was done to show how the molecules of synthetic nanoparticles normally stretch and bend when they vibrate. The fact that metal-oxide nanoparticles stretch in the lower IR region shows that the bonds between the molecules are not extraordinarily strong.

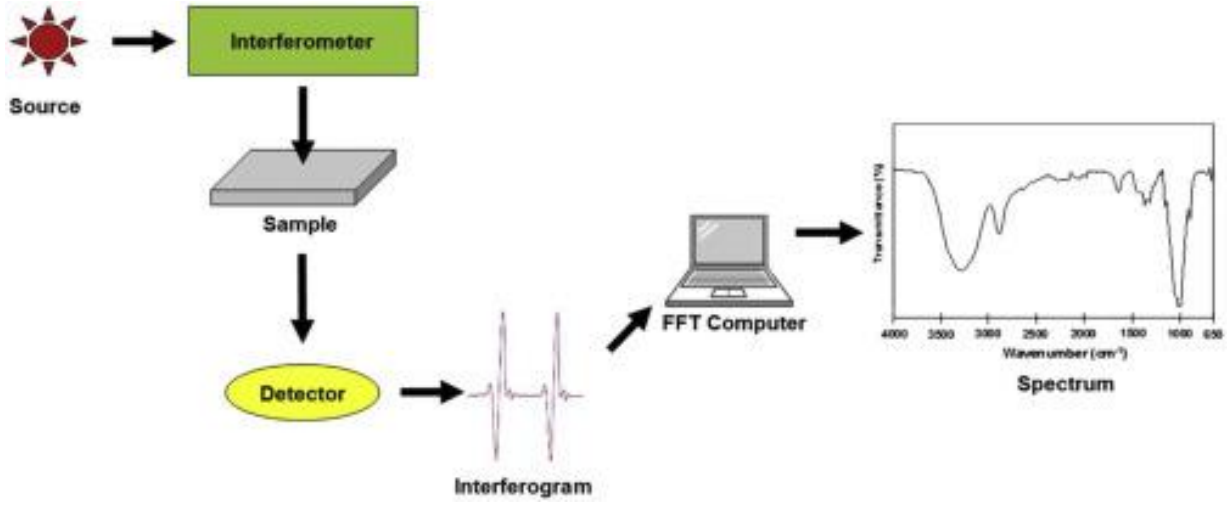

Figure 13 Working Mechanism of FTIR [64].

3.3.3 SEM Analysis

Then SEM was performed with TESCAN MIRA 3. The size and morphology of nanoparticles was determined by the SEM analysis. This analysis was conducted by preparing the glass slides containing the samples of nanoparticles and coating them with gold (30nm) to induce the conduction into the sample under examination. The SEM images show the physical dispersion of the nanoparticles and their varied sizes in nano scale.

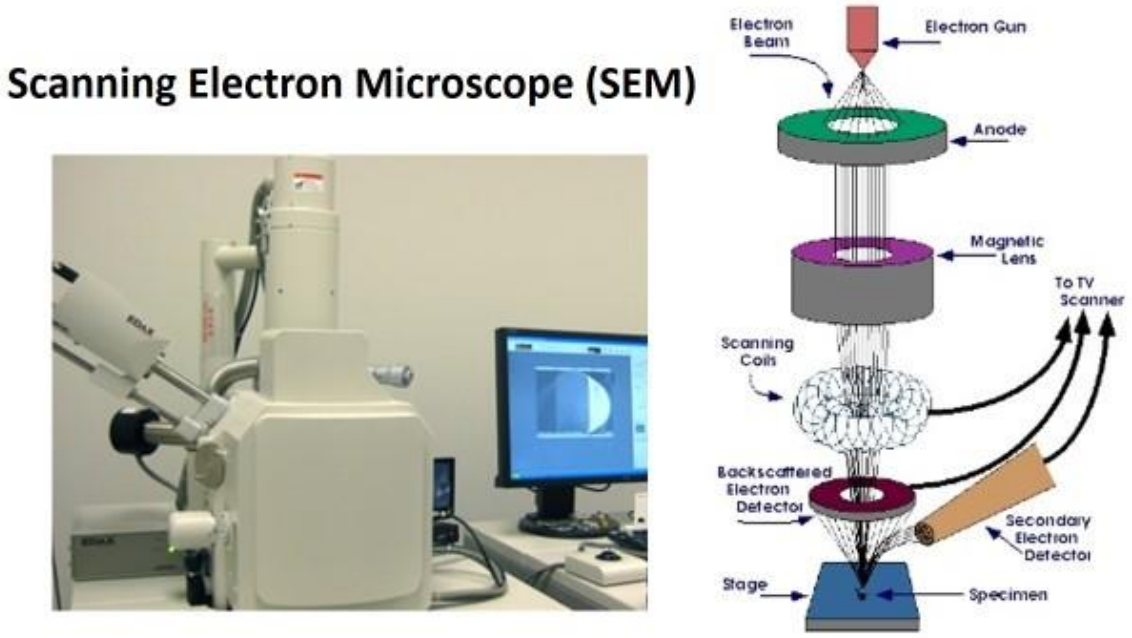

Figure 14 Working Mechanism of Scanning Electron Microscope [64].

3.3.4 EDX Analysis

EDX was performed with SEM TESCAN MIRA 3. This analysis shows the graphical representation of the elemental composition of the nanoparticles and CIP-loaded PVA/GT membrane.

3.4 Contact Angle Measurement

The hydrophilicity of the samples was measured at 25 ◦C using sessile drops method at contact angle analyzer (KRÜSS DSA100) [65]. To determine the water contact angle, 4 L of water was repeatedly dropped onto the film's surface. Each sample was weighed and measured three times. Three tests, on average, yielded this result.

3.5 Mechanical Testing

Wound dressings heavily depend on their mechanical properties to maintain their structural integrity following application onto a wound site. The determination of the ultimate tensile strength was conducted utilizing a state-of-the-art universal testing machine, specifically the LinkamTST350 model. A universal tester (LinkamTST350) with a 200N charge for the cell plus a crosshead rate of 10mm/min was used to calculate the ultimate tensile strength. The samples were crushed between the grips of the testing machine until fissures formed**(Figure 15)**. The force was quantified using millipascals as the unit of measurement.

Ultimate Tensile Strength (MPa)= Force (N)/Area (mm^2)

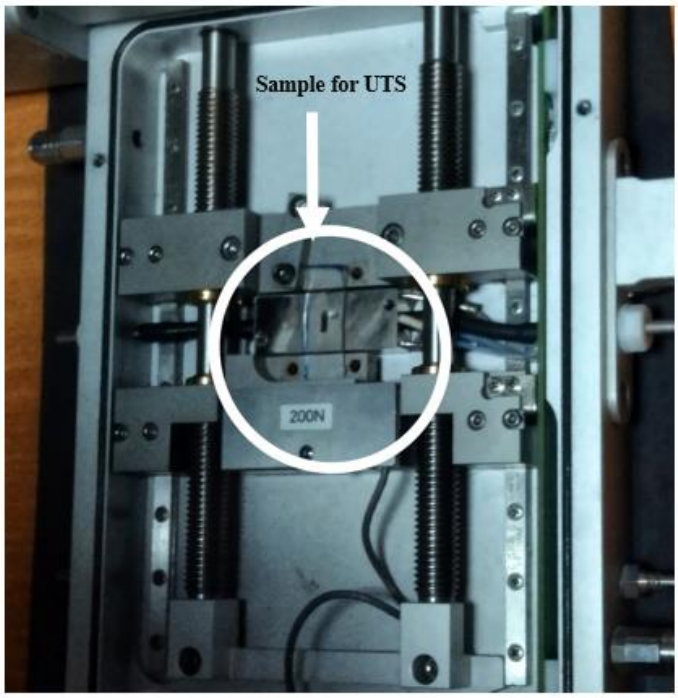

Figure 15 Mechanical Testing of Membrane

3.6 Hemolytic Assay

To determine the harmful effects of the materials (polymeric membranes), a hemolytic test was performed. After obtaining consent, blood samples were obtained from healthy individuals. After letting the blood centrifuge at 19,000 rpm for 10 minutes, it was analyzed.

Phosphate-buffered saline (PBS) was added to the blood cells at a 1:3 ratio after the residue was removed. The samples were kept using a bathtub of water at the temperature of 37 degrees Celsius for 15 minutes after being placed in a PBS solution (10 mL). After adding the diluted blood (0.2 mL), the tubes were inverted and left to incubate for 2 hours. Phosphate-buffered saline (PBS) served as a negative control, while Triton X-100 (1%) served as a positive one. After incubation, the optical density (OD) of the supernatant at 350 nm was measured in all the tubes by centrifuging them for a ten-minute period at 18,000 rpm. The degree of hemolysis was calculated using the following formula.

$$\%\text{Hemolysis} = \text{Sample OD} - \text{Negative Control OD}/\text{Positive Control OD} - \text{Negative Control OD} \times 100$$

3.7 Drug Release Kinetics

The investigation involved an examination of the controlled release of ciprofloxacin from a membrane that had been pre-loaded with Cip. The release process was monitored over a period of 48 hours, during which a predetermined quantity of phosphate-buffered saline (PBS) was introduced. Following the centrifugation process, the supernatant underwent analysis utilizing a UV spectrophotometer. Cumulative drug release was quantified by means of absorbance measurements acquired at a specific wavelength of 273 nm. Moreover, various mathematical models such as zero order model to check the dissolution of drug and first order model to check the release of drug ciprofloxacin from CIP-AuNPs whether it depends on concentration or not were also observed. Higuchi model to check that either the release mechanism of drug CIP from CIP-AuNPs is like the release mechanism from the matrix or not.

3.8 In Vitro Testing

3.8.1 Bacterial Strain Isolation

The study was done with a clinical isolation of *E. coli* bacteria. By mixing samples in deionized water that had been autoclaved, different dilutions up to 10-6 were made. These dilutions were put on Tryptic Soya Agar plates and kept warm for 24 hours at 37°C resulted in growth of several colonies on it. After 24 hours, the grown colonies were picked out and spread out on a selective medium **(Figure 17)** [66]. After isolating the desired types of bacteria, an inoculum was made to test an antibacterial model further.

Figure 16 Agar Plate preparation (Pouring TSA Media in Petri Plates)

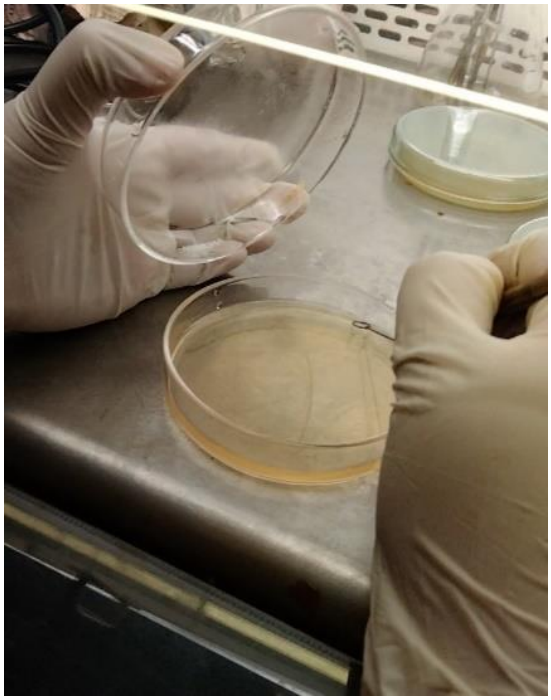

Figure 17 Streaking on Agar Plates.

3.8.2 Antibacterial Testing

The antibacterial activity of CIP-loaded PVA/GT membrane against *E. coli* bacteria was evaluated using the usual well diffusion technique. The culture, which had recently been formed, was carefully distributed onto sterilized nutrient agar plates. To facilitate further experimentation, wells with a diameter of 8 mm were carefully created in the agar plates using a sterilized cork borer. The wells were equipped with polymeric membranes discs solutions and a control was established using 10 micro liters of ciprofloxacin. Following that, the plates were kept at 37 °C for a day to evaluate the antibacterial activity by measuring zones of inhibition (ZOI) surrounding the wells [66].

3.9 In Vivo Testing

3.9.1 Animals

For this experiment, healthy adult rats were used. Each set of rats (n = 4) was kept in its own cage with access to food and water at room temperature. The US Food and Drug

Administration (FDA) issued good laboratory practices in 2010 that governed how rats were cared for and handled [67].

Figure 18 Division of Rats in groups and Acclimatization

3.9.2 Diabetic Foot Ulcer Infection's Induction in Rat Model

Four groups of rats were selected at random. The rats in the control group were not given any chemicals or made diabetic in any way. The other three groups were designed to produce

diabetes outcomes. Nonetheless, one diabetic group served as a control. After the acclimatization of 7 days, Alloxan (140 mg/kg intraperitoneal) was used to induce diabetes in rats, and the animals were monitored for three days. After monitoring them for 3 days, glucose levels were measured with a glucometer, and those below 200 mg/dL were diagnosed as diabetic. The incision wound was created the next day into two groups and infected with *E-coil*; a bacteria known to induce diabetic foot ulcers. Two days later, a swap sample was taken from wound to confirm infection on blood agar plates after observing the site form last two days [68].

Figure 19 Induction of Diabetics

3.9.3 Treatment Design

After confirming an infection, the wound was treated in one treatment group via a Simple PVA/GT membrane while in other treatment group via CIP-loaded membrane, and then monitored for 14 days (as shown in **Figure 20**), during this time the site began to heal. After 14 days of therapy, rats were sacrificed, and histology samples were obtained.

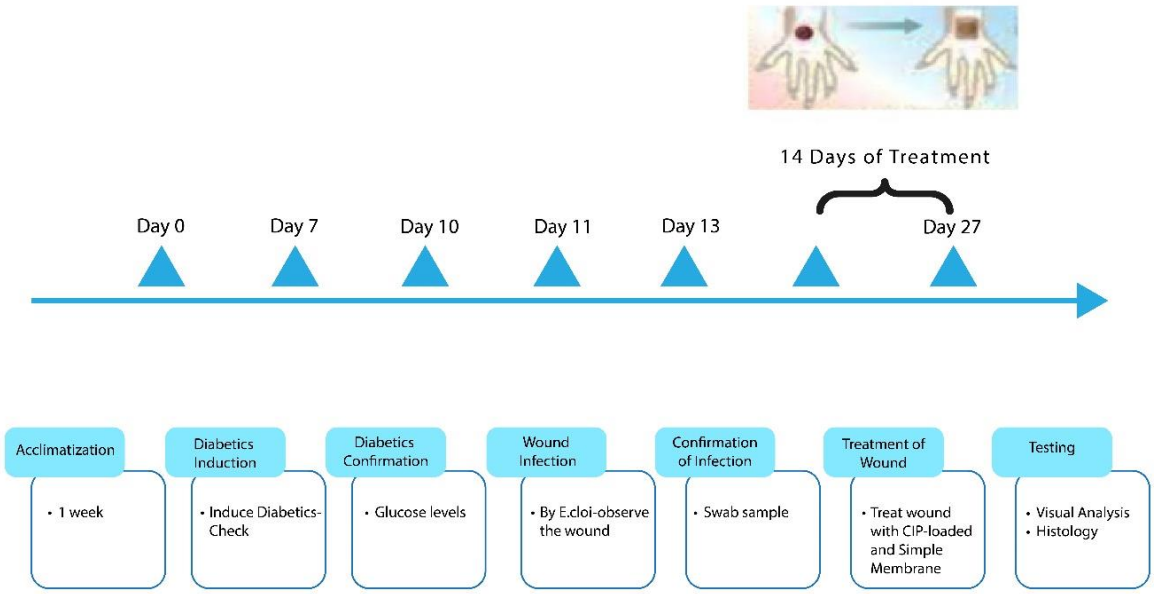

Figure 20 Treatment Design for Diabatic Foot Ulcer Infection

3.10 Statistical Analysis

For the statistical analysis, Microsoft Excel, and GraphPad Prism software (Version 8.0, which is San Diego, CA, USA) were used. Multiple t-tests were used to figure out the p-values.

Chapter 4 Results and Discussion

AuNPs and CIP-AuNPs **(Figure 21)** and fabricated polymeric membranes **(Figure 22)** were successfully obtained and confirmed by different characterizations techniques.

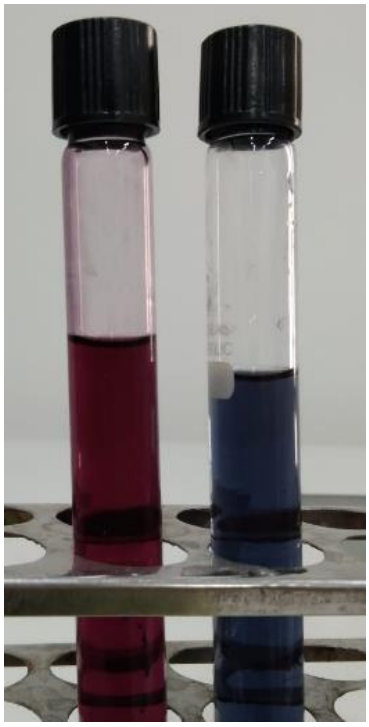

Figure 21 AuNPs(left) and CIP-loaded AuNPs(right)

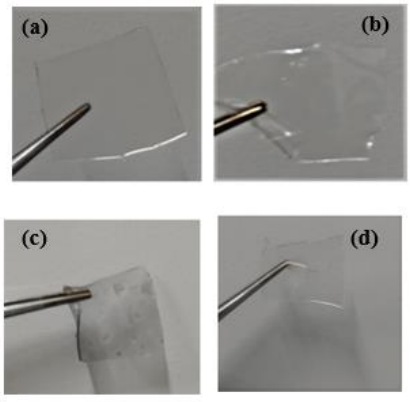

Figure 22 PVA/GT Membranes (a) Pure PVA/GT Membrane (b) PVA/GT Membrane loaded with CIP (c) PVA/GT Membrane loaded with AuNPs (d) PVA/GT Membrane loaded with CIP-AuNPs

4.1 UV–Vis spectrophotometry

Figure 23 shows UV graph plotted between wavelength and absorbance. UV of ciprofloxacin exhibited one peak at 272 nm and the other at 326nm. AuNPs exhibited a peak at 513nm. When AuNPs were loaded with ciprofloxacin the peak shifted to 530nm.

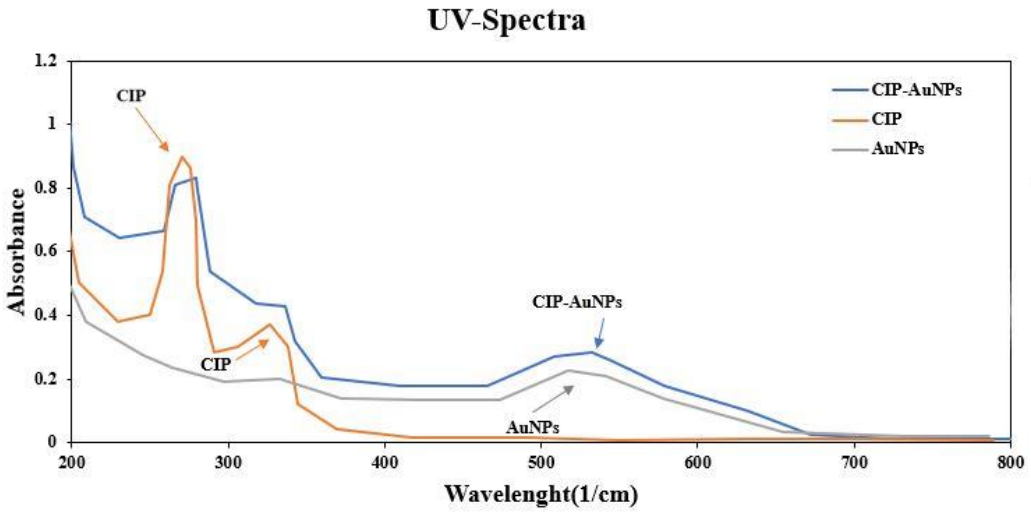

Figure 23 UV-VIS-Spectra of CIP, AuNPs and CIP-AuNPs

4.2 FTIR Analysis

FTIR spectra were collected from the range of 1000 to 4000 cm−1. The FTIR spectrum in the image **(Figure 24)** shows the successful adsorption of ciprofloxacin (CIP) in gold nanoparticles (AuNPs). The characteristic bands of CIP, including the stretching of the N–H bond of the imino moiety on the piperazine group at 3410 cm−1, the primary CIP-AuNPs have a spectrum that includes the amine (N-H) bent of the pyridone moiety at 1644 cm1, as well as the C-F functional group at 1070 cm1. CIP adsorption on the AuNP surface can be verified by observing a change in the wavelength of the N-H stretching band from 3316 cm1 to 3449 cm1. The presence of even a small amount of water causes the O-H stretching band to appear large in the spectrum of CIP-AuNPs. Citrate traces are responsible for the faint bands in the CIP-AuNPs about 2918 cm1.

CIP has been successfully absorbed on AuNPs, as evidenced by the FTIR spectra. The expansion of the O-H stretching band and the movement of the N-H stretching band revealed that CIP was bound to the AuNP surface. The FTIR spectrum also verified the presence of water and trace amounts of citrate in the CIP-AuNPs sample.

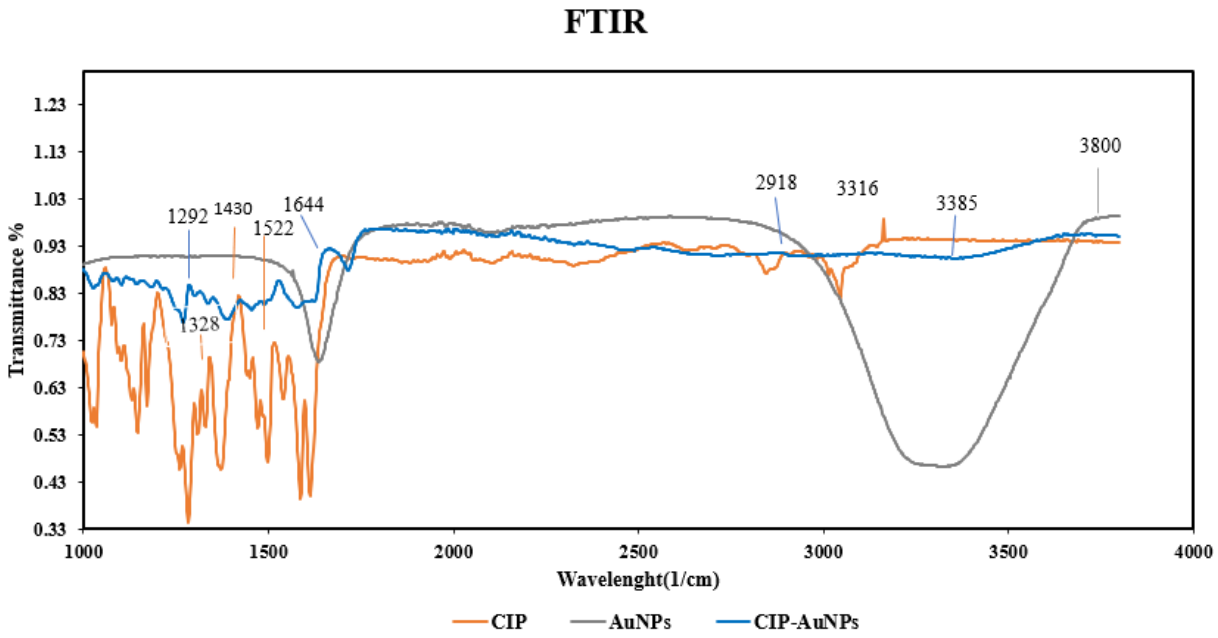

Figure 24 FTIR OF Nanoparticles

4.3 SEM Analysis

To examine the surface morphology of nanoparticles scanning electron microscope was used at 20 kV. SEM images showed that the AuNPs had the shape of spheres with the size of approx. 24nm, and in addition, the SEM examination demonstrated that CIP-AuNPs have a significant degree of polydispersity [60]. **(Figure 25)** PVA has a smooth surface, but it became significantly rougher after the addition of GT and characterized by the presence of white dots on the surface, which can be seen in **(Figure 26).** Furthermore, the SEM study of PVA/GT Membrane loaded with CIP-AuNPs showed that the membrane is saturated with ciprofloxacin gold nanoparticles, the cross section displays a small number of spherical spots as well as fissures. Moreover, membranes had high porosity which is good for the gaseous exchange **(Figure 27)** [70].

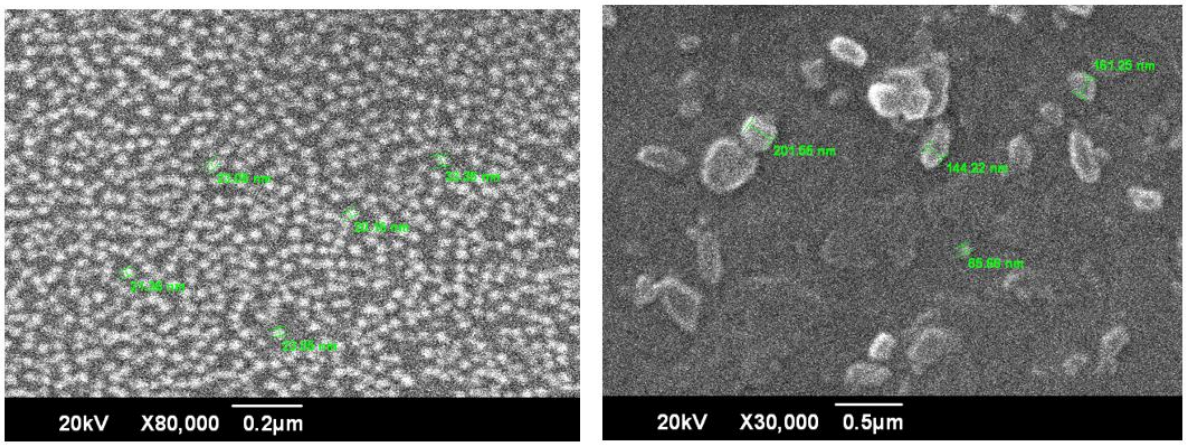

Figure 25 SEM of AuNPs(left) and CIP-AuNPs(right)

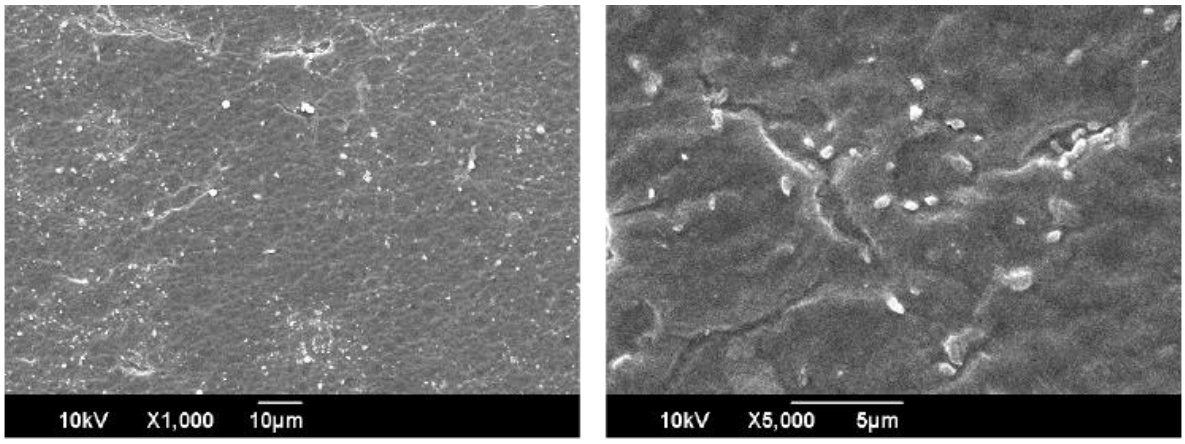

Figure 26 SEM of CIP-loaded PVA/GT Membrane (Surface morphology)

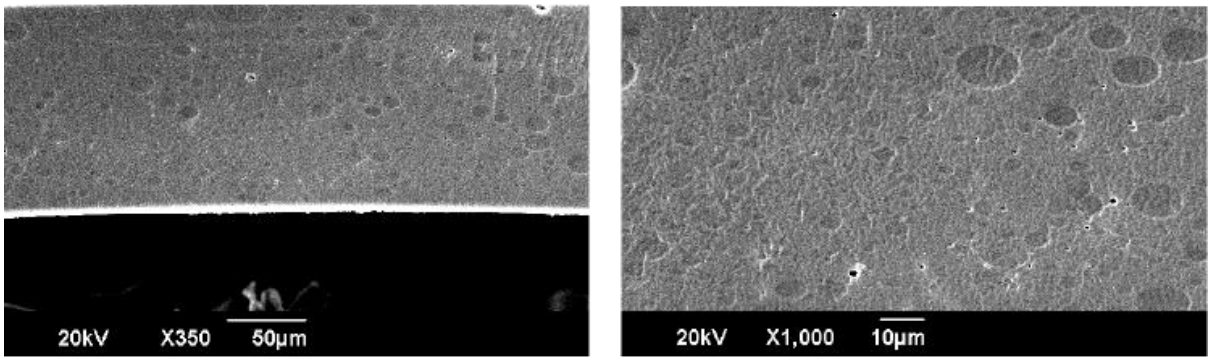

Figure 27 SEM of CIP-loaded PVA/GT Membrane (Cross Sectional)

4.4 EDX Analysis

The accumulation of peaks was displayed on their spectra for the emission of electromagnetic radiation [71]. The results of an EDX analysis showed that gold, along with carbon, was the most abundant element in the sample. EDX analysis was carried out so that elemental mapping could be completed. Because of the unique atomic structure of each component that is present in the CIP-loaded AuNPs **(Figure 28)** and the CIP-loaded membrane **(Figure 29)**, a particular oxygen, chloride, and other components may be distinguished from one another. When the energy was normal, the suitable spectra corresponding to the element Au were found to have substantial between 1.5 and 2 keV.

The EDX spectra (0- 0.5 keV, 2.5-3 keV) obtained from CIP-HCl revealed the presence of chloride as a characteristic of the compound. During the process of making CIP-AuNPs, trisodium citrate was utilised as a reducing and capping agent. This is what causes the Na peak to appear at 1–1.5 keV in the energy spectrum. The percentage composition of carbon and oxygen in CIP-AuNPs was found to be higher when compared to earlier spectra of AuNPs obtained using EDS. Because the mounting base was made of silicon, there was a significant overlap between the Si peaks that occurred between 1.5 and 2 keV.

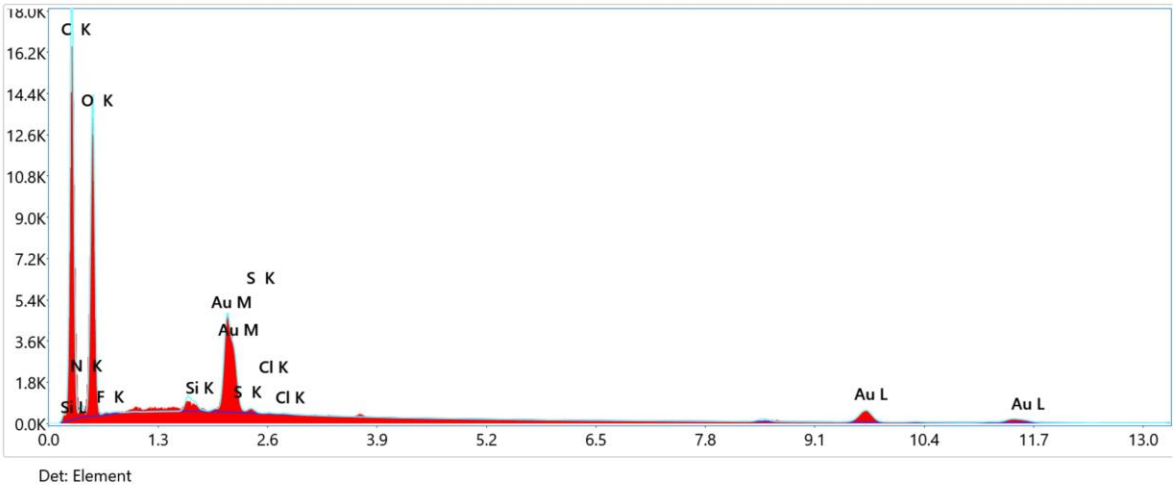

Figure 28 EDX of CIP-AuNP

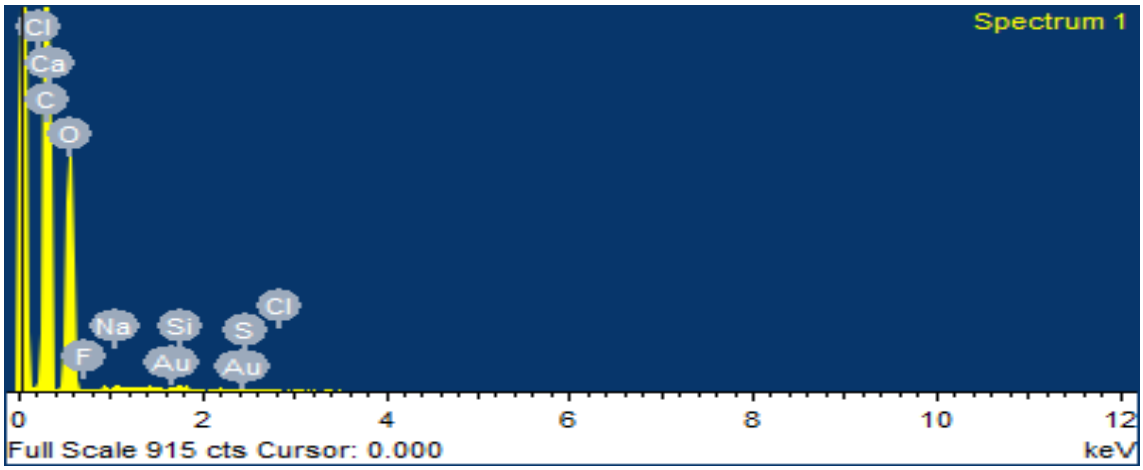

Figure 29 EDX of CIP-loaded PVA/GT Membrane

4.5 Contact Angle Measurement

These membranes have high water absorption capacity, according to the data. Hydrophilicity is measured by the water contact angle. The greater the hydrophilic characteristics of a material, the lower its contact angle [65]. **Figure 30** shows the water contact point of the membranes. All water contact angles were found to be smaller than 90 degrees, as shown in **Figure 31.** This means that the membranes were hydrophilic.

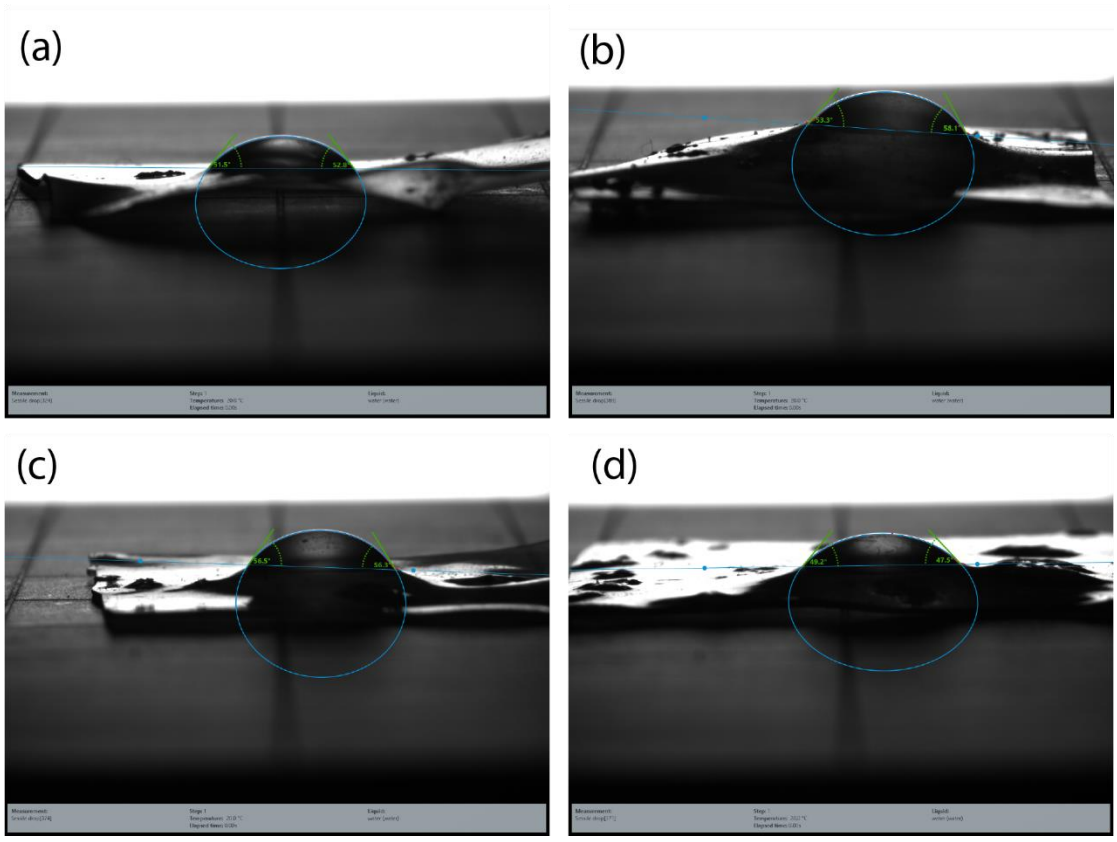

Figure 30 Contact Angle Measurement of PVA/GT. Membranes (a) PVA/GT Membrane (50.1°C) (b) PVA/GT Membrane – CIP (55.3°C) (c) PVA/GT Membrane - AuNPs (57.77°C) (d) PVA/GT Membrane loaded with CIP-AuNPs (47.12 °C)

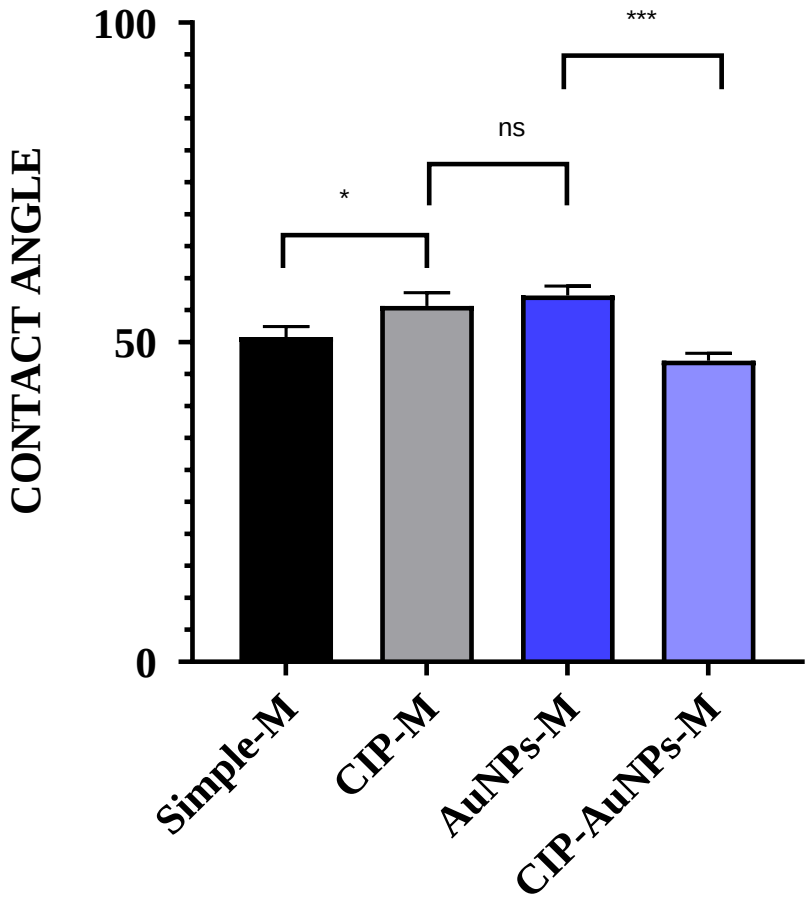

Figure 31 Contact Angle Measurement of Simple Membrane(PVA/GT) and PVA/GT Membranes Loaded with CIP, AuNPs and CIP-AuNPs(CIP-M, AuNPs-M, CIP-AuNPs) with p <0.005flagged (**) that shows statistically signification between them and p > 0.005 flagged (ns) statistically insignificant results.

4.6 Mechanical Testing

The Ultimate tensile strength of Simple PVA/GT membrane and CIP-AuNPs (CIP-loaded) WAS 3.88 and 4.31, respectively. Tensile Strength was in the range of human strength which is 4.38 ± 0.038MPa [54]. This shows that fabricated membrane (CIP-loaded) has a positive impact on wound healing **(Figure 32)**.

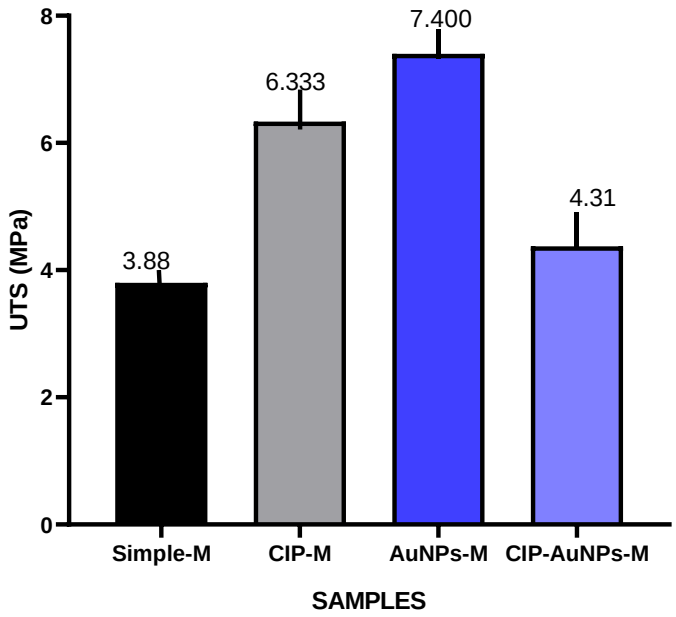

Figure 32 Ultimate Tensile Strength of Simple (PVA/GumM) and PVA/GT Membranes Loaded with CIP, AuNPs and CIP-AuNPs(CIP-M, AuNPs-M,CIP-AuNPs)

4.7 Hemolytic Assay

The OD, or optical density, of the supernatant was measured using UV-Vis spectroscopy at 545 nm for quantitative analysis. The degree of hemolysis was determined by comparing it to the threshold for hemolytic activity set by the ASTM F756 standard, which is 5% [72], it was determined that none of the samples evaluated possessed any such activity. Hemolysis was confirmed to be 100% using a Triton X-100-treated blood positive control. Hemolytic activity was zero in the negative comparison (PBS solution containing blood). Whereas simple (PVA/Gum) Membrane, CIP-M, AuNPs, which M, and CIP-loaded M demonstrated 3.6%, 2.6%, 3.3%, and 4% hemolytic activity, respectively during testing. **(Figure 33)**

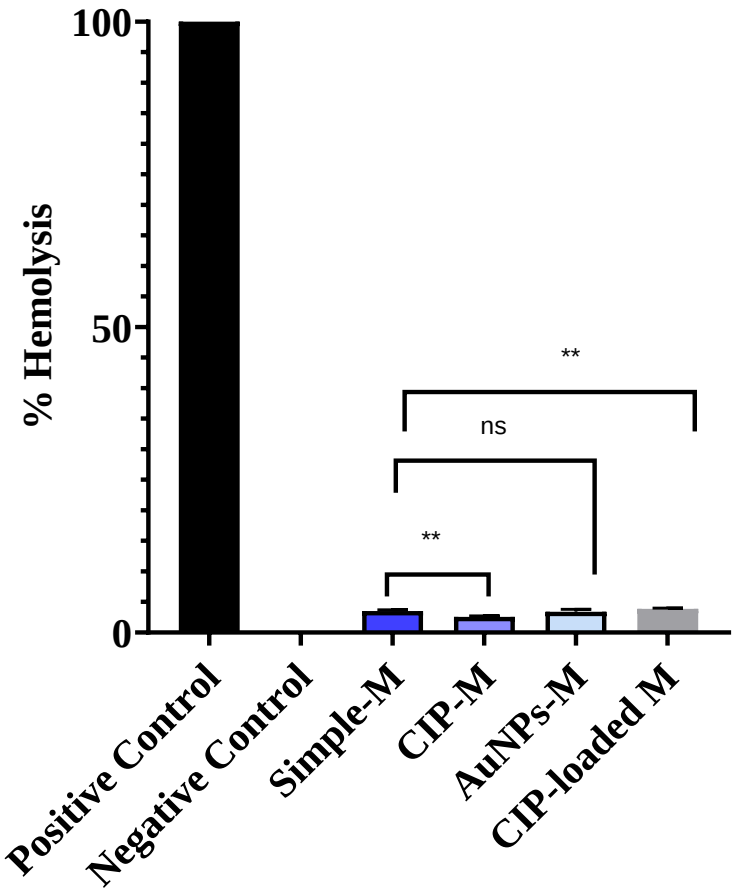

Figure 33 Hemolytic Assay of Simple Membrane (PVA/GT.) and PVA/GT Membranes Loaded with CIP, AuNPs and CIP-AuNPs(CIP-M, AuNPs-M,CIP-AuNPs) with p <0.005flagged (**) that shows statistically signification between them and p > 0.005 flagged (ns) statistically insignificant results.

4.8 Drug Release Kinetics

Figure 34 depicts a medication release curve with two distinct phases (typical biphasic drug release pattern). In the initial phase till 5 hours formulation demonstrated fast CIP release that could last up to five hours. This indicated that CIP effectively absorbed to the surface of AuNPs. Due to their small dimensions, CIP-AuNPs possessed a high surface-to-volume ratio, allowing for rapid release after compression. The loading dose provided by the initial explosive release

will aid in preventing the spread of the disease, while the ongoing release stage will enhance the therapeutic effect [73]. **Figure 35** shows the kinetic models of drug release.

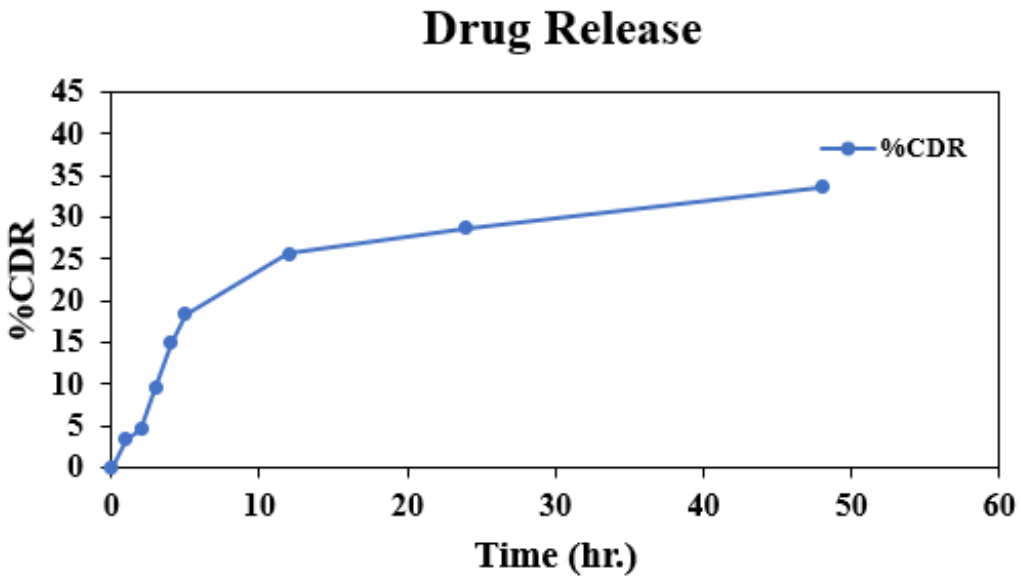

Figure 34 Drug Release of CIP

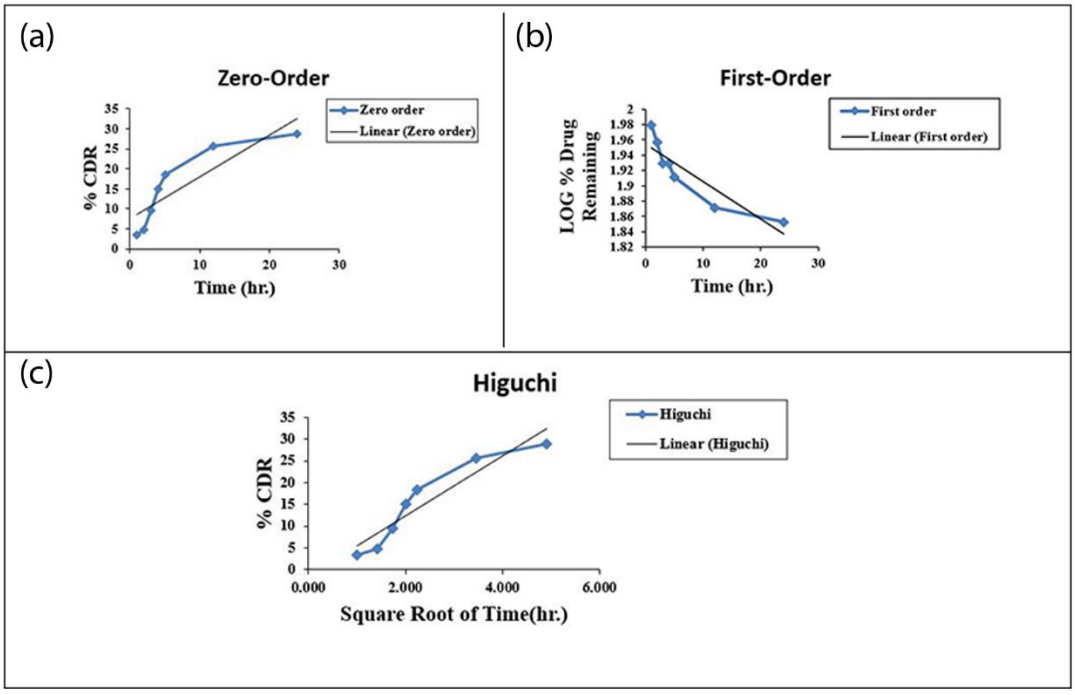

Figure 35 Kinetics Models of Drug Release (a) Zero Order (b) First Order and (c) Higuchi

4.9 In Vitro Testing

4.9.1 Antibacterial Testing

The zone of inhibition recorded for the CIP-AuNPs membrane was 23 mm, which is an improvement over the 21 mm recorded for the CIP membrane. However, when AuNPs were added to the PVA/GT membrane, no inhibitory zone was formed. Patients with ulcers from diabetic feet benefit from the antibacterial action of the membrane encapsulating CIP-AuNPs **(Figure 36,37)**

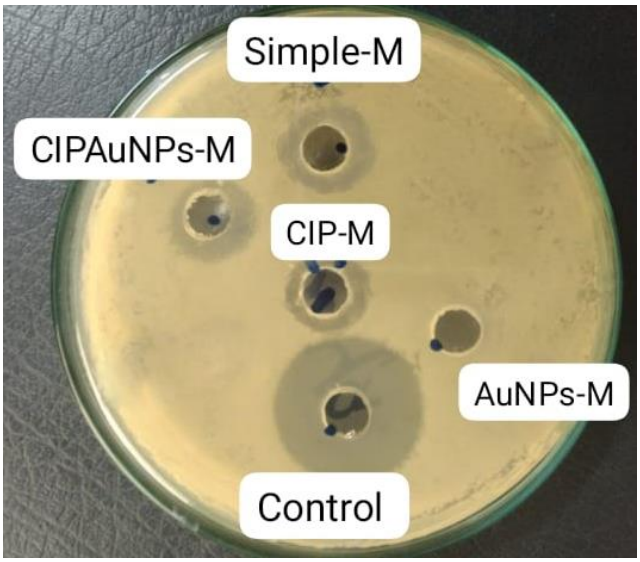

Figure 36 Antibacterial Testing of Membranes having ZOI 21mm for CIP loaded PVA/GT Membrane and 23mm for CIP-AuNPs loaded PVA/GT Membrane.

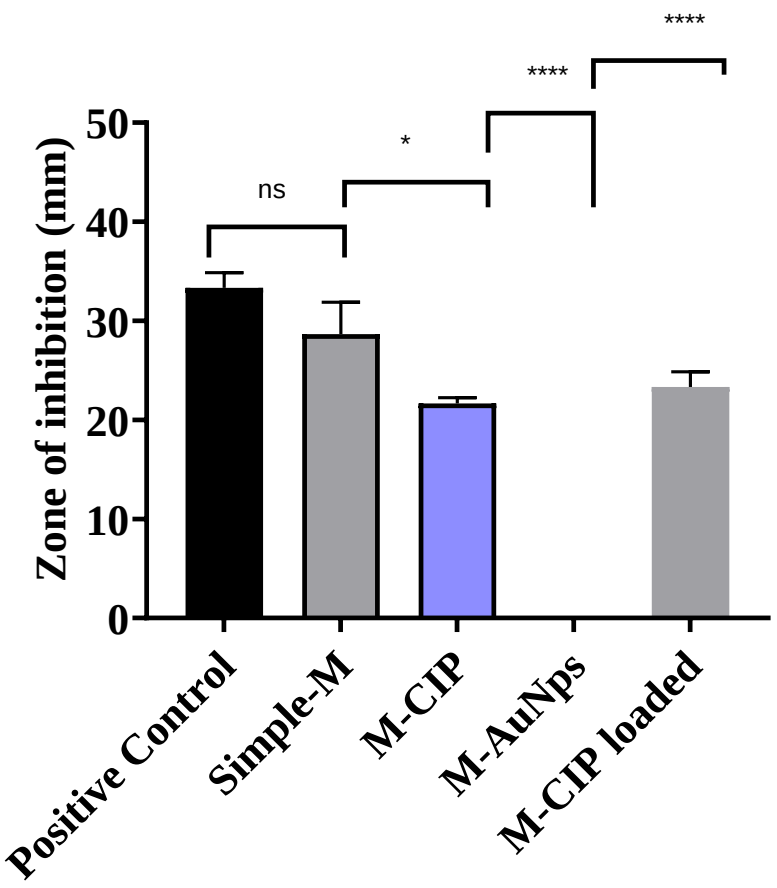

Figure 37 Zone of Inhibition of Membranes shows statistically significance results p<0.005 flagged (***) and have certain p value but less than p flagged (**)

4.10 In Vivo Testing

4.10.1 Animals

Rats were successfully acclimatized for 7 days and taken care of by the FDA rules.

4.10.2 Diabetic Foot Ulcer Infection's Induction in Rat Model

Using a glucometer, the induction of diabetes was confirmed as the glucose level was below 200**(Figure 38)**. **Figure 39** depicts the effective swapping of a control sample from the foot skin of a normal rat and a sample from an infected wound caused by DFU infection from the infected

group on blood agar plates. Colonies grew on the infected sample's agar plate, and a colony was then transferred to the blood agar plate for the confirmation of infection, which was confirmed by the appearance of large, thick, greyish-white, opaque disc-like colonies, indicating that *E-coil* successfully infected the DFU wound **(Figure 40).**

Figure 38 Confirmation of Diabetes by using Glucometer.

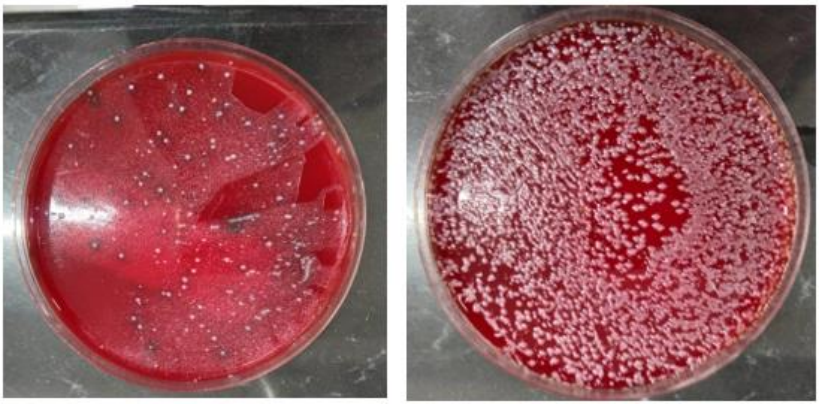

Figure 39 Control(left) and DFU (right) Confirmation on Blood Agar Plates

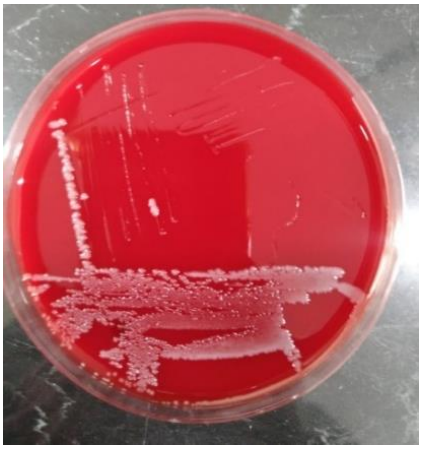

Figure 40 *E-coil* Growth on Blood Agar Plate

4.10.3 Treatment Design

Simple Membrane and CIP-loaded Membrane were successfully applied on the wounded areas of DFU and were successfully observed for 14days **(Figure 41)**

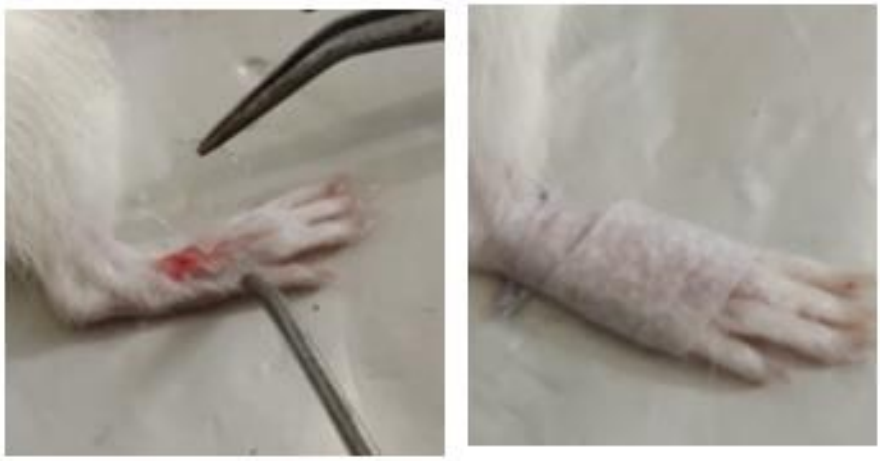

Figure 41 CIP-AuNPs loaded PVA/GT Membrane Applied on Wound.

4.10.4 In Vivo Testing

Figure 42 Shows the in vivo testing results in the form of histology and visuals. (a) shows histological examination of the section reveals a fragment covered with keratinized stratified squamous epithelium with underlying dermis showing pilosebaceous units and subcutaneous tissue showing blood vessels, fat and smooth muscle.(b) shows histological examination of the section reveals a fragment covered with keratinized stratified squamous epithelium with

underlying dermis showing hemorrhage and dense inflammatory infiltrate comprising of neutrophils, lymphocytes and macrophages. It shows that infection on foot of diabetic rat has been successfully induced. In (c) wound was treated with PVA/GT membrane then histological examination of the section reveals a fragment showing keratinized stratified squamous epithelium with underlying dermis showing granulation tissue and moderate inflammatory infiltrate comprising of neutrophils and lymphocytes. In (d) wound was treated with CIP-AuNPs loaded PVA/GT membrane and histological examination of the section reveals a fragment showing keratinized stratified squamous epithelium underlying granulation tissue formation and mild inflammatory infiltration.

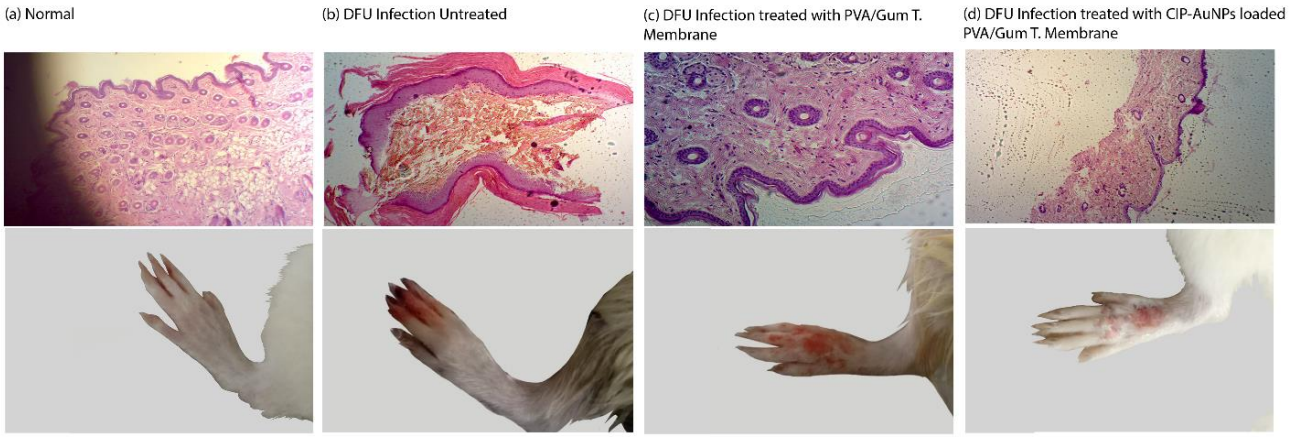

Figure 42 (a) Normal (b)DFU (c) DFU treated with Simple Membrane (PVA/Gum) (d) DFU treated with CIP-AuNPs loaded PVA/GT Membrane.

Chapter 5 Conclusion

5.1 Conclusion

In conclusion, prepered PVA/GT membrane loaded with CIP-AuNPs shown promising properties for diabetic wound healing. The membrane has high mechanical strength and minimal hemolytic activity, indicating that it might be used as a wound dressing. The addition of AuNPs and ciprofloxacin increased the membrane's antibacterial activity, as seen by the suppression of *E. coli* growth. These findings imply that the PVA/GT membrane loaded with CIP-AuNPs has the potential to treat diabetic foot ulcers and enhance wound healing outcomes.

Chapter 6 Limitations and Future Prospective

CFU analysis should be quantified viable microorganisms and assessed microbial growth in a sample. Research on microorganism prevalence and abundance relies on this method. This method helps identify pathogenic bacteria and fungi, identifying infections and assessing antibiotic susceptibility.

In this research, membrane adhesion to wounds hinders sample collection and healing. To improve membrane adherence, we must investigate and develop new adhesive technologies. Structural design optimization can boost productivity and patient comfort. Before integrating these advances into healthcare, thorough clinical trials must prove their safety and efficacy.

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