Effective Delivery of Doxycycline and Epidermal Growth Factor for Expedited Healing of Chronic Wounds.

BY

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ABSTRACT

The problems and high medical costs associated with chronic wounds necessitate an economical bioactive wound dressing. A new strategy was investigated to inhibit MMP-9 proteases and to release epidermal growth factor (EGF) to enhance healing. Doxycycline (DOX) and EGF were encapsulated on polyacrylic acid modified polyurethane film (PAA-PU) using Layer-by-Layer (LbL) assembly. The number of bilayers tuned the concentration of DOX and EGF released over time with over 94% bioactivity of EGF retained over 4 days. A simple wound model in which MMP-9 proteases were added to cell culture containing fibroblast cells demonstrated that DOX inhibited the proteases providing a protective environment for the released EGF to stimulate cell migration and proliferation at a faster healing rate. In the presence of DOX, only small amounts of the highly bioactive EGF are sufficient to close the wound. Results show that this is new and promising bioactive dressing for effective wound management.

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STATEMENT OF ORIGINALITY

The work presented in this thesis is, to the best of my knowledge and belief, original, except as acknowledged in the text, and the materials have not been submitted, either in whole or in part of a degree at this or any other university.

Abhilash Kulkarni

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LIST OF ABBREVATIONS

Chemicals and biological terms:

CH Chitosan

DOX Doxycycline

EGF Epidermal growth factor

MMP Matrix metalloprotease

CWF Chronic wound fluid

AWF Acute wound fluid

ECM Extra cellular matrix

PU Polyurethane

AA Acrylic acid

PBS Phosphate buffer saline

Instrumentation and methods

AFM Atomic force microscopy

UV Ultra violet

FTIR Fourier transform infrared spectroscopy

ELISA Enzyme linked immunosorbent assay

MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)
-2-(4-sulfophenyl)-2H-tetrazolium)

LbL Layer by Layer assembly

CHAPTER 1

Introduction

1.1 Wounds and their classifications

Human skin is a complicated organ composed of ectodermal tissue, whose major function is to form an effective barrier between the body (inside organs) and the physical, chemical and biological environments. Skin prevents water loss through evaporation and also prevents the penetration of exogenous substances from the environment. When there is a breach in the integrity of this tissue layer, a wound is created and the barrier is compromised. Thus a wound is defined as physical injury that disrupts the continuity of tissue structure and its functions. [1]

The classification of wounds into acute and chronic is based upon the total healing time and repair process. Acute wounds are those that heal completely in a definite time frame, generally within 12 weeks, leaving minimal scarring. Knife cuts, wounds caused by abrasion, surgical cuts wounds, burns due to corrosive chemicals, and exposure to radiation are all acute wounds. Chronic wounds may take a long time to heal (6 months and longer), since healing does not go through the orderly set of stages. [2, 3] Chronic wounds ultimately fail in wound closure. Wound healing is hindered due to poor treatment, physical and biological state, nutritional status and prolonged infections. Diabetic leg ulcers and sores, venous statis, and pressure ulcers are generally classed as chronic wounds.

1.2 Chronic wounds and wound healing paradigm

Chronic wounds are a worldwide health problem and significantly increase healthcare costs. In United States, approximately 15 percent of diabetic patients develop skin ulcers, [4] resulting in more than 60,000 amputations per year. The costs associated

with the care of skin disease or wound care are very significant. Direct medical costs are as much as $116 billion annually for patient in United States. [5] In Manitoba the direct cost of treating diabetes is estimated to be $ 250 million. [6]

Wound healing is a complex and dynamic process of tissue repair that involves cells, local biochemical factors such as growth factors, components of extracellular matrix (ECM), neutrophils, and macrophages, and proteases interacting with each other. These interactions are responsible for the activation or deactivation of bioactive molecules, which in turn regulate the cellular activities responsible for tissue repair. [7] Tissue repair undergoes a series of biological stages that are often overlapping and classified into different phases - haemostasis or coagulation, inflammation, migration and proliferation, and finally remodelling. [8-10]

Haemostasis or Coagulation and Inflammatory Phase

Wound healing begins when platelets contact a damaged blood vessel and begin to adhere to the exposed collagen. Once the platelets adhere, vasoconstriction begins to decrease blood flow to the damaged capillary blood vessels. Fibrin promotes platelet adhesion, which in turn release clotting factors, calcium ions, ADP, and ATP. [11, 12] Once the clot dries it acts as a provisional extracellular matrix (ECM) allowing cells to migrate to the injured site and providing strength to the injured tissue. These platelets secrete wound healing mediators such as growth factors. The growth factors in turn activate macrophages and stimulate a cellular response such as cell differentiation, cell growth and cell proliferation. Chemotactic factors and various other mediators are released by parenchymal cells. [9] In response to the chemotactic factors, neutrophils are the first to

arrive at the wound site. The main function of the neutrophils is to cleanse the wound site by eliminating any foreign particles, dead cells and micro-organisms. Therefore, neutrophils are found in large numbers during the first 2 or 3 days following an injury. Neutrophils decline in numbers and monocytes are attracted to the injured site within 2 days. The monocytes differentiate into pro-inflammatory macrophages by adhering to the extracellular matrix. As shown in **Figure 1.1**, macrophages are activated and release different growth factors, matrix metalloprotease (MMP) and cytokines that are mainly responsible for the formation of new tissue. These macrophages also destroy bacteria and cleanse the wound. [7]

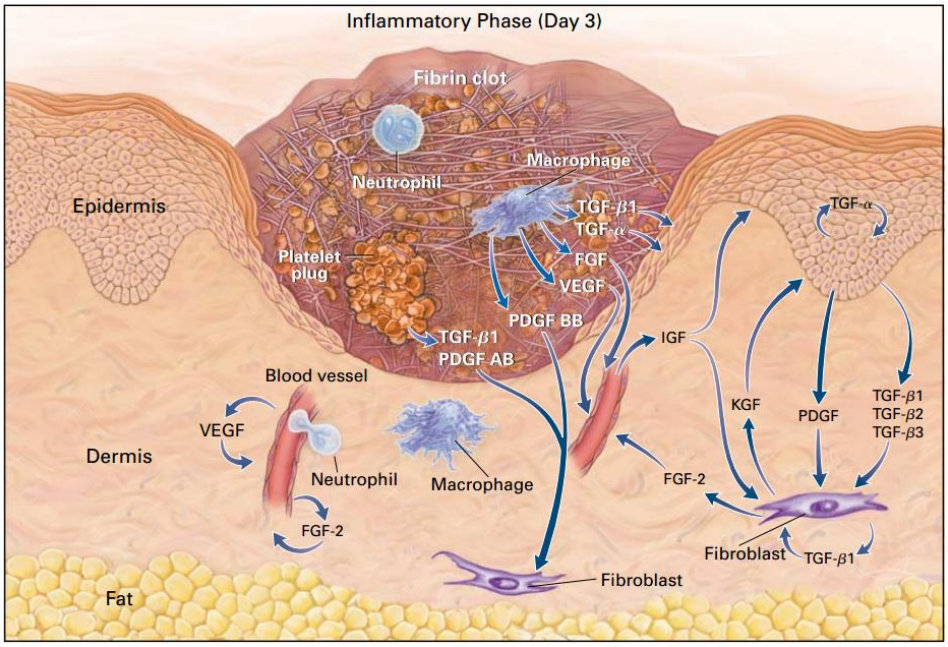

Figure 1.1. Wound healing - Inflammation stage "Reproduced with permission from (Singer, 1999)".

Migration and Proliferation Phase

During the migration and proliferation phases, epithelial cells and fibroblasts start to move to close the wound, and new granulation tissue is formed replacing the previously formed provisional matrix. Fibroblasts synthesize collagen, which eventually replaces the fibronectin and fibrin of the previously formed ECM. New ECM is produced by fibroblasts, which in turn facilitates the migration of additional fibroblasts. As shown in **Figure 1.2**, during angiogenesis new granulation tissues are formed with new capillaries and blood vessels. Growth factors and reactive oxygen species (ROS) in low concentrations stimulate angiogenesis with the establishment of blood flow. [8]

Remodelling Phase

Cellular connective tissues are formed and the new epithelium cells are strengthened determining the final scar during the remodelling phase. Type III collagen in the newly formed blood vessels is finally replaced by collagen type I. [13] The net collagen content is balanced by its synthesis and degradation rates. In the dermis, 70-80% of collagen is available forming tight cross-links to other collagen and protein molecules, and increasing the tensile strength of the scar by 20%. [14, 15] Synthesis of collagen III to collagen I provides the necessary structure and strength, but degradation of the collagen III depends upon many factors such as various MMPs produced by macrophages, and fibroblast cells. The scars thus formed can change in appearance over time, from several months to 2 years.

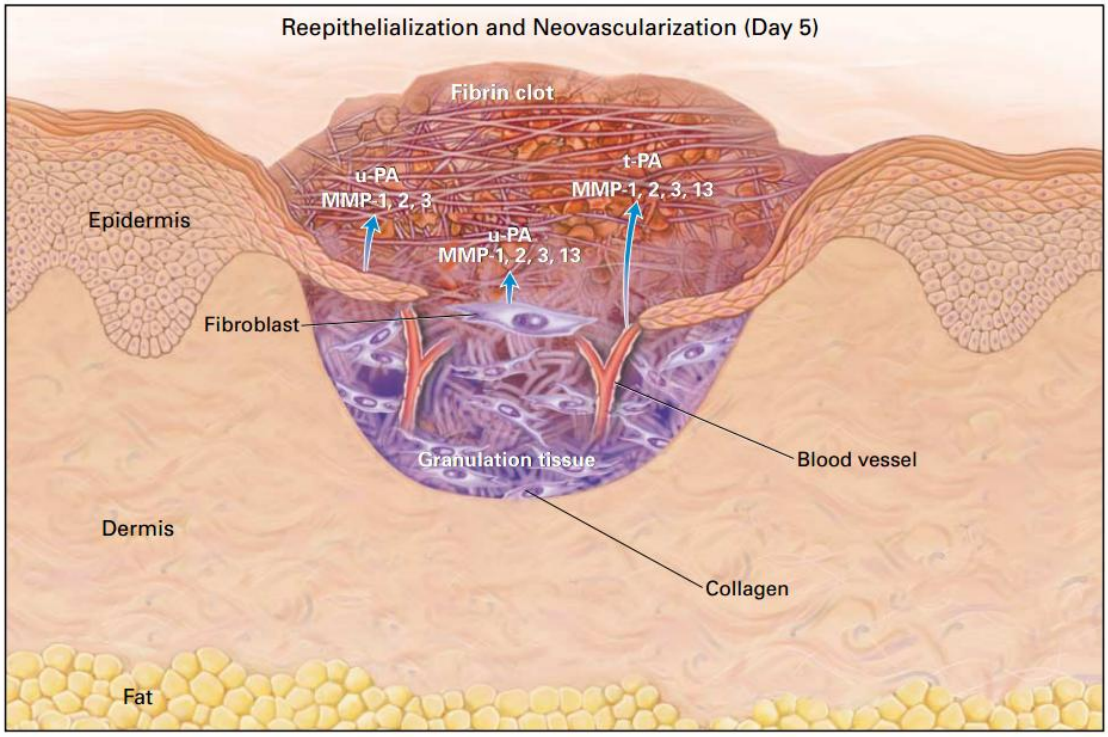

Figure 1.2. Wound healing after day 5. "Reproduced with permission from (Singer, 1999)".

1.3 Impeded wound healing and chronic wounds

Healing of wounds is delayed when the processes of healing do not occur in sequence. Wounds that take more than a couple of months to heal fall into the category of chronic wounds. Many factors influence the healing process and can impede the process of healing. These factors are classified as local and systemic. [16-17] Local factors have a direct relationship with the wound itself, such as bacterial contamination (infection), tissue maceration, availability of oxygen, presence of a foreign body, and irregular cellular activities. [16] Systemic factors are more diverse and are based on the patient's overall health history and current state of health. [17] A patient's healing ability may be affected by systemic factors such as nutrition, stress, renal disease and age.

The healing of chronic wounds is often hindered by stricken granulation tissue formation and increased fibrosis in the inflammatory stage, [18] since the increased fibrosis does not allow keratinocytes to migrate for re-epithelialisation and the wound edges do not close. This is due to the disruption of protease (tissue destructive protinease enzyme) regulation, leading to an excess amount of proteases in these wounds. Several researchers have evaluated the proteolytic activity in acute and chronic wound fluids (CWF) and have found that chronic wound fluids showed proteases elevated 30-60 fold compared to acute wound fluids (AWF). [19, 20] This elevated level of proteases degrades growth factors and protease inhibitors, and upsets the formation of the ECM, prevents the migration of keratinocytes and finally destroys the newly forming tissues. Even with good wound care, a 20-week healing rate of diabetic foot ulcers was only 31%. [21] The elevated amount of protease is primarily responsible for stalling the healing of chronic wounds at the inflammatory stage: protease degrades the extracellular matrix, destroys the newly formed tissues and prevents the attachment and migration of keratinocytes. [22, 23]

1.4 Protease activity in chronic wounds

During the inflammatory stage of normal wound healing, all the processes of removal of necrotic tissue and debris, control of bacterial infection, and activating fibroblasts to move will occur. Neutrophils are present for only 72 hours in acute wounds, but are present throughout the healing stage in chronic wounds. [24, 25] The excessive amount of neutrophils leads the way to elevated levels of proteases. [26-30] Proteases are a group of enzymes found in a variety of micro-organisms. These enzymes are small in size, with compact spherical structures that catalyze the cleavage of peptide bonds in proteins. [31] Depending on their catalytic mechanisms and functional groups at the active

site, proteases are classified into 3 categories, namely, MMPs (Matrix metalloprotease), serine protease, and cysteine protease. Cysteine proteases are also known as thiol protease. The serine in serine protease serves as the nucleophilic amino acid at the active site. Serine protease is found in excessive amounts in chronic wounds. The abundance of spatially distributed MMPs is not balanced by [26] the tissue inhibitor of matrix metalloproteases (TIMPs) which are very low in number or absent in chronic wounds. [27, 32, 33] This imbalance in the ratio of MMPs to TIMPs is responsible for the destruction of the extracellular matrix due to the excess MMPs. [20] As these proteases degrade various growth factors, they suppress the mitogenic activity of cells in the chronic wound site, and delay the wound closure.

MMPs are a large family of zinc-containing endopeptides having the ability to break down connective tissue. Based upon the structure, 20 different types of MMPs may be categorized into different subclasses within the family of the MMPs. These subclasses are collagenases, gelatinases, stromelysins, and membrane type MMPs. [34, 35] Elevated levels of collagenase protease (MMP-1, MMP-8) and gelatinase protease (MMP-2, MMP-9) were observed in chronic wounds. [26, 28, 36, 37] Reports have shown that the gelatinase protease (MMP-2, and MMP-9), when present at significantly higher activity levels than in normal wounds, impedes the healing process - since gelatinase protease, particularly MMP-9, can break down collagen (Type I-IV) into small peptides. [38] Liu et al. found that the concentration of MMP-9 in diabetic ulcers was 8-10 times higher than the normal concentration of MMP-9. [39] Moor et al. also found that in venous ulcers the MMP-9 was 7 fold higher [40] and Rayment et al. showed that the MMP-9 activity was 25 times higher in chronic wound fluid when compared with control. [41]

MMP-9 was the major factor responsible for stalling wound healing. Overall, clinical results have shown that wounds remain unhealed when large amounts of the growth factor degrading MMP-9 were found at the wound sites.

1.5 Recombinant molecules promoting wound healing

Frequently used compounds for skin wound healing in microparticulate forms include growth factors, CH (chitosan), collagen, hyaluronan, and beta-Glucan. [42] The use of recombinant molecules in chronic wounds offers interesting and challenging possibilities to modulate the microenvironment. However, the outcome of clinical studies to date demonstrates that an important and often underestimated aspect of the growth factor wound healing paradigm is the effective delivery of polypeptides to the wound site. Growth factors are used for tissue regeneration and can promote wound repair (healing). During the healing process, the cell-to-cell interactions and cell-to-matrix interactions are all controlled by a wide variety of growth factors. Different types of growth factors have been tested on animal models, and have been found to influence the healing process either directly or indirectly. As shown in **Figure 1.3,** these growth factors include platelet derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VGEF), insulin-like growth factor (IGF-1), transforming growth factor (TGF-b1), and granulocyte-macrophage colony stimulating factor (GM-CSF). [7, 43-45]

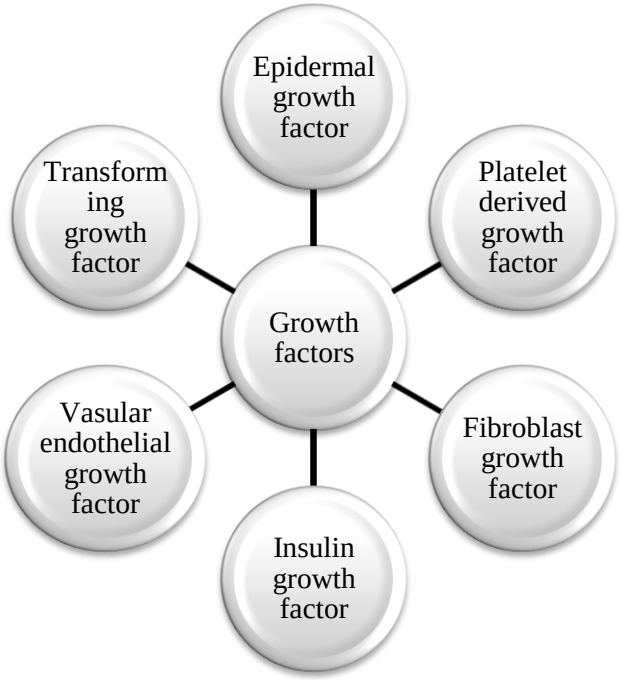

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Figure 1.3. Different Types of Growth Factors

In 1962, Stanley Cohen discovered human epidermal growth factor , a protein with 53 amino acid residues, a molecular weight of 6045 Da and 3 interamolecular disulfide bonds. Of the various different growth factors, EGF is most important because it plays a vital role in keratinocyte differentiation and stimulates cells to proliferate and migrate. Specifically, EGF has high affinity receptors in both keratinocyte and fibroblast cells. [46-49] EGF controls the cellular activities in the healing process including migration, stimulation and proliferation of cells, synthesis of RNA, DNA, proteins and hyaluronic acid, and matrix substances necessary for healing. Clinical results show that these epidermal growth factors increased in the granulation tissue formation and its organization in 7 days, increased collagen content by 50% and protein content by 33%, thus enhancing the wound healing process. [49, 50]

However, the physical instability (polymerization of monomer into dimer and trimer by disulfide exchange) and the prevalent chemical reaction (deamidation of the asparagine residue) are major problems associated with EGF. [51-53] The half-life of this growth factor in the body is too short, about 1 hour, to exhibit any biological effects when applied via free forms, ointments or injections. [53, 54]

Moreover, samples from chronic wound fluid had significantly higher levels (almost 47 fold) of degraded EGF compared with acute wound fluids, [19] indicating the absence of EGF in the wound site. This is due to the fact that the proteases in the wound easily decompose the EGF, [55] leading to impaired healing as there is no EGF to express the mitogenic activity of cells. Studies conducted on a wound model *in vivo* using Sprague-Dawley rats also demonstrated that long-term exposure to epidermal growth factor is required for complete cellular response, [49] and needs to be facilitated by a controlled release system to avoid frequent dressing change. Frequent dressing changes can increase the risk of nosocomial infection [56] and may increase patient pain, high economic cost involved and also increase the nursing costs and the potential for delayed wound re-epithelialization. In short, encapsulation of growth factors is needed for protection from degradation caused by high protease levels. Sustained release is needed to provide longer exposure time to EGF cells to achieve mitogenic activity.

CHAPTER 2

Currently-Available Dressings for Chronic Wounds

2.1 Assessment of the Wound and Choice of Appropriate dressings

Before a wound dressing is chosen, a comprehensive wound assessment should be made considering the type of wound, its depth (or thickness), its size and shape, its location and the amount of exudates. [57] These factors play a significant role in the choice of dressing. Different dressings are available to treat different stages of a wound. Some dressings can be applied directly, in cases such as full thickness wounds, the wounds must be cleansed and operated on before applying the dressing.

Once the wound is assessed, the appropriate dressing should be easy to use, economical, provides patient comfort, provides a moist environment, protects the wound from dehydrating, and allows gaseous exchange. Proper wound management is a combination of understanding the wound healing process and a complete awareness of the properties of different available dressings. For example, different occlusive dressings are used depending upon the amount of exudates in wounds. Occlusive dressings such as hydrocolloid and hydrogel dressings are used when the wound contains light to moderate exudates. In contrast, a foam dressing consisting of porous polyurethane sheets is applicable for partial to full thickness wounds containing moderate to heavy exudates.

2.2 Modes of Treatment

Different types of treatments are available currently to expedite the healing of chronic wounds. Different aspects of wound care management and wound bed preparation are involved. Management of exudates and necrosis (debridement) is often done by the use of primary dressings, vacuum assisted closure (VAC) and high compression bandages. Other approaches involve reducing bacterial inflammation. Since

chronic wounds exhibit higher bacterial infection, many practitioners use different antibacterial agents in the dressings to reduce the bacterial contamination thereby accelerating the wound healing. Another practice of wound management is the use of biological species such as growth factors, cells, components of ECM such as CH, collagen and bioengineered skin substitutes.

2.3 Summary of Currently Available Dressings

The concept of dressing a wound was recorded in 2100 B.C. Wound management has been practiced from the time of Egyptians, as far back as 1400 B.C. where three healing gestures were used. Wounds were washed with beer and hot water and covered with dressings composed of herbs, ointments and different oils. [58] Egyptians treated open wounds using different natural components in pastes composed of grease, honey and lint. [59, 60] A compositions of Two-thirds grease and one third honey decreased the bacterial count (*E. coli and Staphylococcus sp.*) from 10^5 to 10^2 within a day of application. [61] Around 400 B.C. ancient Greeks used a piece of tin pipe to drain surgical puss. Later, in 280 B.C., a Greek barber developed a syringe called "pyulkos", to inject liquids into the human system and suck puss from wounds. [59]

Infection of wounds and the associated formation of puss were due mainly to *Staphylococcus* bacteria. Puss formation displayed the body's ability to heal itself. In 1865, Joseph Lister demonstrated the treatment of wounds using dressings dipped in carboxylic acid, an antiseptic. In 1880, Joseph Gamgee developed the now famous composite dressing made of cotton or rayon enclosed in a retaining sleeve. These dressings were used with iodine or phenol to reduce bacterial infection and were also

used as desloughing and debriding agents. [44] The term debridement was first described by Reyher, and later practiced by Antoine Depage. The early work of Winter et al. brought many advances in the field of wound dressings such as use of films, foams, gels, CH and polysaccharide materials [62] that are all designed to be occlusive to liquids such as wound exudates.

Different types of occlusive dressings such as films, hydrocolloids and hydro gels are now available. [63] Hydrocolloid dressings absorb wound exudates and convert them into a jelly-like mass. These dressings are available in the form of sheets and films or as composite dressings in combination with other materials such as alginates and are used for wounds that have moderate exudates. Calcium sodium alginate, a hydrocolloid dressing, has outstanding absorption properties and haemostasis ability. [64] Hydrogel dressings absorb and retain water. Since they enhance autolytic debridement they are suitable for cleansing dry dead tissues and are used for light exudate wounds. [65] The major difference between hydrogel and hydrocolloid dressings is seen in their mechanisms. A hydrogel dressing physically entraps liquid, whereas, a hydrocolloid dressing absorbs the exudates and forms a hydrophilic gel.

Apart from occlusive dressings, different biological dressings composed of biomaterials play a significant role in expediting the healing process. Biological dressings made of collagen films and collagen gels stimulate the formation of fibroblasts, thus enhancing the migration of endothelial cells. Bilayer wound dressings consist of an elastic external layer, generally polyurethanes and silicones serving as an artificial epidermis to protect the wound, and an inner layer of hydrogel that acts as a substitute for the dermis, providing a basic support to proliferate the cells. The earliest bilayer wound

dressing, composed of an inner layer of collagen/chondroitin-6-sulfate sponge attached to a silicone membrane was developed by Yanan in the 1980s.

In case of full thickness wounds, surgical operations are carried out to debride and cleanse the wound before the application of dressings. Skin substitutes are the latest development in wound dressings for diabetic ulcers. These are biodegradable, tissue-engineered wound care dressings cultured with skin cells. [66] These skin substitutes feature anatomic and mechanical properties [44] that degrade and leave behind connective tissues. Skin substitutes consist mainly of collagen, human keratinocytes, and dermal fibroblasts. **Table 1** indicates commercially available skin substitutes.

Table 1. Commercially available bioengineered skin substitutes

Name of skin substitute	**Manufacturer**	**Type**	**Description**	**Uses**
Integra™	Integra LifeScience (Plainsborough, NJ)	Acellular artificial skin	Bilayer wound dressing composed of collagen/Chondroitin-6 sulphate matrix on a temporary silicone sheet	Both deep and partial thickness burn wounds.
Apligraf™	Organogenesis (Canton, MA)	Cellular-allogenic, epidermal and dermal skin substitute	Bilayer dressing containing type -1 collagen mixed in a suspension of dermal fibroblast cells.	Treatment of venous and chronic foot ulcers and also for burn

				wounds.
Dermagraft™	Advanced Tissue Sciences (LaJolla, CA)	Cellular-allogenic, dermal skin substitute	Cultured human fibroblasts on a biodegradable polyglycolic acid or polyglactin mesh, cells metabollically active at grafting stage.	Full thickness chronic foot ulcers.
Biobrane™	Dow Hickham/Bertek Pharmaceuticals (Sugar Land, TX)	Acellular	Silicone membrane bonded to nylon fabric, collagen.	Wound covering for Partial thickness burns
Alloderm™	Lifecell Corporation (Branchberg, NJ)	Acellular dermal graft	Normal human dermis with all the cellular material removed, rehydrated before grafting.	Dermal template for grafting to burns and other wounds
Epicel™	Genzyme Biosurgery (Cambridge, MA)	Cellular-autologus,	Autologous keratinocytes cultured from patient skin biopsy	Permanent wound close for burn wounds.
TransCyte™	Advanced Tissue Sciences	Cellular-allogenic	Allogeneic neonatal foreskin fibroblasts	Used for deep and full

			seeded on nylon mesh; freeze-dried to kill cells and preserve dermal matrix.	thickness burn wounds.

The major disadvantages of these skin substitutes are: they biodegrade rapidly, with no synchronized dermal regeneration rate; [67] their uses are limited to completely infection free wound sites. [44] They are expensive per application; and have a limited shelf life. [44]

Apart from biological and traditional dressings a new series of medicated dressings have been designed which contain anti-bacterial agents, depending upon on the requirement of the wound. Several researchers have found that the absence of growth factors, particularly EGF, decreases the cellular response, thereby impeding the healing process. Many researchers have applied EGF topically to wounds and have found that such topical application of EGF has accelerated wound healing. [68-70] As EGF has a relatively short half life of about an hour in vivo and is physically instable, studies conducted on a wound model in vivo using Sprague-Dawley rats also demonstrate that long-term exposure to EGF is required for complete cellular response, [49] and needs to be facilitated by a controlled release system to avoid frequent dressing change. In short, growth factors need to be encapsulated for the purpose of protection from degradation and sustained release.

Many research groups have encapsulated growth factors into various biomedical materials, including nanofibers [72, 73] and microspheres [74-76] to facilitate sustained delivery.

The basic concept of EGF encapsulation began in 1985 when Buckley et al. encapsulated EGF into pellets that were in turn embedded into a polyvinyl alcohol sponge. [49] The pellets released 10-20 µg of EGF per day. The released EGF promoted an increase in the organization of granulation tissue and a 50% increase in collagen synthesis, [49] indicating that compared to daily injections of EGF, long term exposure improved the healing process in rats by increasing the mitogenic activity. Later, Sheardown et al. released EGF from a semi-solid carbopol gel carrier system on a corneal epithelial wound on New Zealand white rabbits. [77] The healing rate was significantly faster with a treatment of EGF for a period of 8 hours. Ulubayram et al. designed a novel bilayer wound dressing containing EGF in gelatin microspheres as instrument for sustained release of EGF into the wound locality. The wound area decreased significantly in several days depending on the dose of EGF released. Park et al. showed that 63.1 ng of EGF was released from the nano-particles in 167 hours, causing a significant stimulation of cell proliferation compared to free EGF. [78] The regular release of EGF from the nano particles stimulated continuous DNA synthesis. Dogan et al. designed dextran hydrogel that was used as a vehicle to deliver EGF and bFGF into Wistar albino rats. At the third day after implanting the hydrogels onto the rats, they observed capillary sprouts; no capillary sprouts were observed in empty hydrogels. On the seventh day, higher levels of angiogenesis was observed clearly indicating that the released EGF accelerated the wound healing in rats. EGF released from PCL-PEG nano-fibers showed enhanced keratinocytic expression in keratinocytes when compared to controls. [80] Scheinder et al. developed a self assembling peptide (SAP) nanofiber scaffold in combination with epidermal growth factor. [81] The wound healing rate was evaluated using a human skin equivalent tissue model. They

reported that the released EGF accelerated the wound to cover by 5 fold when compared to controls with scaffolds, and by 3.5 fold compared with the scaffold with EGF.

Apart from releasing growth factors to wound sites and expediting the healing process by expressing the mitogenic activity of cells, many researchers have worked on reducing or inhibiting the excessive amount of proteases. By inhibiting proteases, a clean environment is provided for growth factors to function. Generally the levels of proteases in chronic wounds are 30-60 times higher when compared to acute wounds. [19, 20] Many researchers have used different compounds to inhibit the protease. These include oleic acid, [46] higher concentrations of gelatin, [82] oxidized regenerated cellulose, [83, 84] and tetracyclines. Some dressings are also available commercially. These include Promogran® a product of Johnson and Johnson. [85-87] Promogran® is a matrix composed of oxidized regenerated cellulose and collagen. By absorbing exudates, the matrix can form a soft biodegradable gel that inactivates MMPs through physical binding. FIBRACOL® is another commercially available dressing which is made of 10% alginate and 90% collagen. This product also reduces MMPs activity. [88] Dermax® a dressing containing a mixture of metal ions and citric acid, reduces free oxygen radicals and levels of MMP-2 in vitro. [89]

As stated in section 1.4, MMP-9 is the protease that is highly elevated in chronic wounds. Commercially available dressings inhibit MMPs by absorbing wound exudates. Many other research groups have worked on doxycycline - a broad spectrum antibiotic belonging to the family of tetracycline. Researchers have shown that this tetracycline can inhibit MMPs in both clinical studies and *in vitro*. [90-93] The levels of MMP-9 are 25 times higher in chronic wounds than in acute wounds. [41] Several studies have shown that

doxycycline decreased MMP-9 significantly. [94-98] Other researchers have released doxycycline from hydrogels or nanofibers to suppress the activity of MMP-9. For example, Anumolu et al. showed that doxycycline can be released from PEG hydrogels and inhibit MMP-9, showing a superior wound healing response on NM-exposed skin when compared to controls. [99] Feng et al. designed a PLLA nanofiber scaffold containing doxycycline and proved the hypothesis that doxycycline can be released in a controlled manner from these biodegradable scaffolds for up to 6 weeks. [100] In another case, gelatin microspheres were developed and cross linked with EDC as a vehicle to carry doxycycline and release it in a controlled fashion. [101] According to Chin et al., levels of MMPs were reduced by a daily topical application of 1% doxycycline gel. [102] The controlled delivery of drugs, including biological species to a wound site over a prolonged time would be beneficial since it reduces the need for frequent dressing changes. Therefore, to encapsulate doxycycline and control its release profile would be advantageous to enhance the *in vivo* efficacy.

2.4 Drug Delivery Systems

Typically, drugs have been administered into the human system in conventional forms such as pellets, ointments, drops, intravenous solutions and creams. [102] However, the growing need to deliver complex drug molecules including different types of proteins, necessitates complex novel delivery systems to improve the efficacy and the safety of the drugs to the human body. Since the 1970s, drugs have been encapsulated and delivered through various polymers. A major advantage of using polymers is the ease of handling and the ability to control their physical and chemical properties. These polymeric

materials release drugs by different mechanisms: diffusion, chemical reaction and solvent activation.

1. Diffusion

The release rate of diffusion is characterized by the drug migrating from a core, or its initial position, through the polymeric system leading out to the polymer's outer surface and finally to the body. There are two different types of diffusion systems: (i) matrices and (ii) reservoirs. [102] The process of diffusion is generally described by a series of equations governed by Fick's first law of diffusion.

Through the matrix device, the drug is dispersed homogenously throughout a polymer. Matrix diffusion system before release (time =0) and after partial drug release (time = t). In a reservoir device, a core of drug is surrounded by a polymeric membrane. The nature of the membrane determines the rate of release of the drug from the system.

2. Chemical Reaction

The release of the drug is facilitated by a chemical reaction either through polymer degradation or by chemical cleavage of the drug from the backbone of polymer. [102] The drug is chemically or enzymatically cleaved to assist its release.

3. Solvent Activation.

The release occurs as a result of swelling of the polymer or osmotic effects. [102] The release behaviour of the encapsulated drugs through above methods is affected by several factors such as speed of degradation of polymer, pore size, and thickness of

encapsulating shells. The amount of drug loaded, however, is very limited due to the size and shape of the capsules.

In 1992 Decher initially developed a very simple, versatile and inexpensive method - Layer-by-Layer (LbL), deposition to produce multilayers in a nano scale within a structure. The basic principle of this technique is the alternate deposition of polyelectrolytes with opposite charges based on physiochemical interactions. The major advantages of LbL in the biomedical field are its capability to load different biomolecules on films and fibers, the ability to control the order and location of multiple polymer layers in nanoscale, and its versatility. This system also provides control over the biomolecules in the design of its loading capacity, and its robustness under physiological conditions. This type of encapsulation maintains the biological activity of its bio-molecules. Many trials have been made using this technique both in biomedical applications and in other fields. For example, in order to produce a cytocompatible interface, LbL technique was used on synthetic polymers to develop layers of ECM components like gelatin, collagen, and chondroitin sulfate. [103] The LbL technique has already been used to encapsulate fibroblast growth factor. [104-105] However, the use of LbL is rarely found to deposit epidermal growth factors onto different substrates for release in healing wounds.

CHAPTER 3

Purpose, Objectives, and Hypotheses

The increase in the number of diabetic patients over time heightens the chances of developing a skin ulcer and high medical costs involved in their treatment. The process of healing a skin ulcer is delayed from couple of months to years as the process is stalled at the inflammatory stage. This is due to the imbalance in the tissue inhibitors and proteases. Only 31% of healing rate of diabetic foot ulcers was observed in a 20 week healing rate despite with good wound care. [21] Several factors influence the healing phase of a skin ulcer including the patient's history.

Once the skin ulcer is developed, huge amounts of money is invested per patient for treating the ulcers as well as the pain which they experience during the healing process. Research in the field of wound healing management is extensive because of the medical cost involved and the prolonged healing time. Wound healing management evolved since the time of the Egyptians who used honey and grease to dress wounds. Nowadays, wound dressings include the use of antibacterial and biological agents to render healing in the forms of anti bacterial dressings, or occlusive dressings.

The major turn over in wound management was the use of growth factors. The use of recombinant molecules in chronic wounds offers interesting and challenging possibilities to modulate the microenvironment surrounding the wound. Several researchers started to apply growth factors topically in the form as ointments, injections daily. However the presence of excessive proteases degrades the growth factors. Long term exposure of EGF to cells is needed to achieve mitogenic activity of cells. In order to obtain the mitogenic activity of cells, researchers started to release the growth factors from nano-fibers, or microspheres which provide a continuous exposure of growth factors to cells leading to increased mitogenic activity of cells. This helped to expedite the

healing process. On the other hand, some researchers have used different kind of protease inhibitors to inhibit the proteases to create a safe environment for the growth factors to perform their functions with being degraded by the proteases.

It is now clear that two different approaches - controlled release of EGF and use of protease inhibitor - DOX, have been used by researchers to expedite the healing process. Different type of encapsulation technique that is used by the researchers has always endangered the growth factors in terms of its activity and also by the presence of excessive proteases. A rationale to start investigations on the use of both protease inhibitors and growth factors using an LbL technique to provide sustained release of EGF and DOX into the wounds to expedite healing. And to characterize its construction process, understanding the potential interactions to maximize the effective usage of these biological species.

The purpose of this research is to develop a novel dressing which is capable of releasing DOX - a protease inhibitor which inhibits MMP-9 proteases, thereby protecting later released EGF from massive proteases and simultaneously delivering EGF for effective wound closure.

The specific objectives of this thesis are to:

a) To Design a strategy to expedite the healing process by encapsulating both EGF and a protease inhibitor - DOX, on functionalized surface polyurethane (PU) film.

$$(CH/DOX)_5 + (CH/EGF)_{20}$$

$(CH/EGF)_n$ = n layers of CH and n layers of EGF

b) To encapsulate the EGF and doxycycline with a couterion - CH by using a Layer-by-Layer assembly. This technique is very simple, versatile, does not involve harsh treatment conditions, and allows the researcher to control the release of EGF and DOX for certain days into wound sites.

c) To monitor the release of the encapsulated DOX and EGF.

d) To quantify the bioactivity of the released EGF in comparison with native EGF.

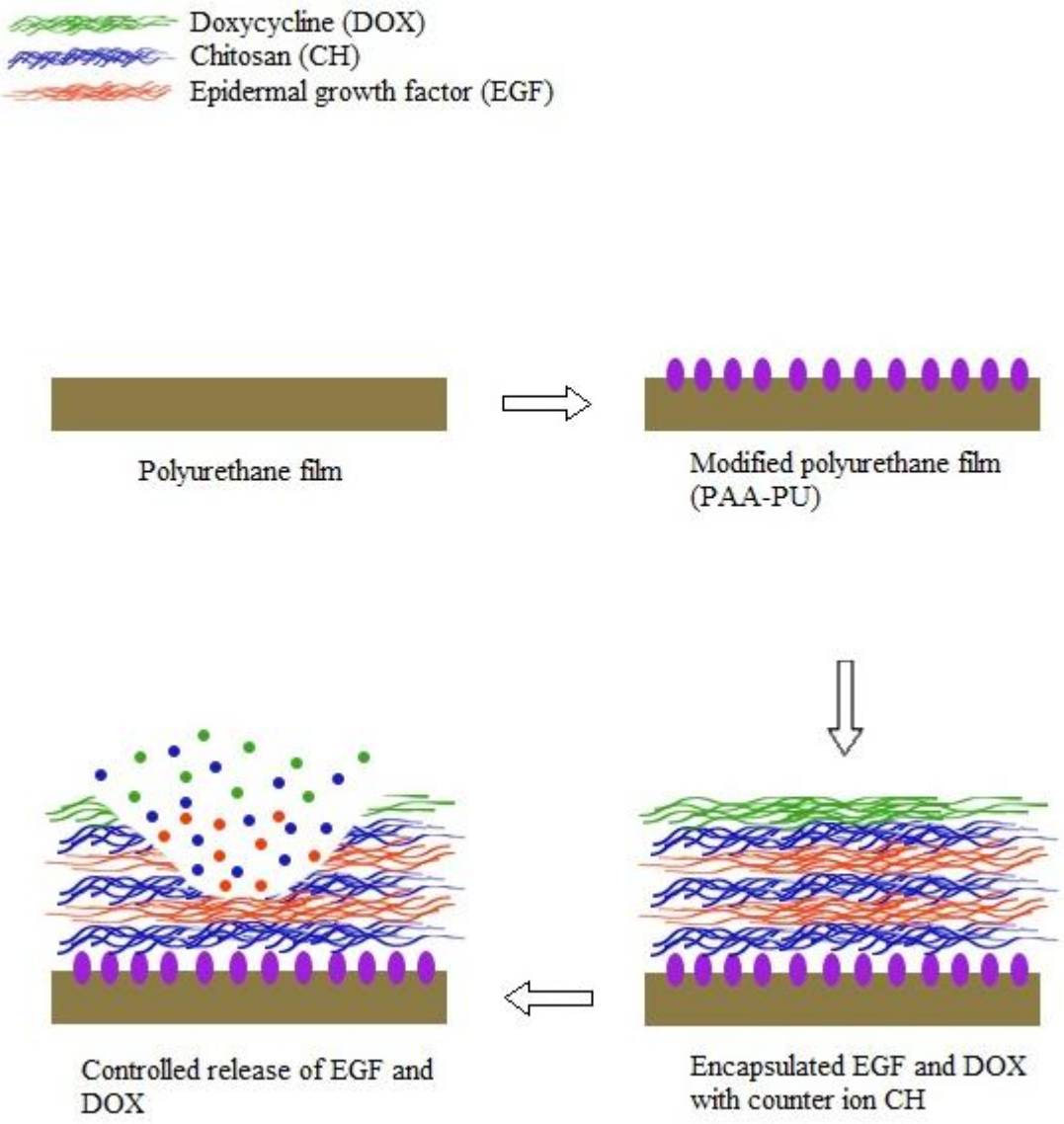
e) To evaluate in-vitro wound healing promotion of the developed dressings in a simple wound model.

f) To evaluate cytoskeletal organization of actin filaments in fibroblast cells by F-actin staining.

The rationale of this thesis was based on following points:

(1) We hypothesize that layer by layer assembly can be used to encapsulate small proteins such as epidermal growth factors. We have found that Layer by layer assembly is used to encapsulate and release big proteins such as BSA, DNA, and fibroblast growth factors. We hypothesize that the encapsulated epidermal growth factor can be released with well retained bioactivity.

(2) Addition of doxycycline on top of previously deposited CH/EGF is designed to release doxycycline first and inhibit MMP-9 protease and then release EGF in the absence of proteases. We hypothesize that the released doxycycline can inhibit MMP-9 proteases, providing a safe environment and protecting the later released epidermal growth factor.



Schematic Diagram 1: Schematic representation of experimental design and methodology.

CHAPTER 4

Materials and Methods

In order achieve the objectives of this research; the experimental design is basically divided into two parts:

Part 01: Layer by layer assembly of EGF on PAA-PU film.

Part 02: Layer by layer assembly of DOX + EGF on PAA-PU film.

The first part is designed to encapsulate and controlled release EGF only and in the second part a protease inhibitor - doxycycline is encapsulated on top of previously encapsulated EGF (Part 01).

4.1 Materials

Polyurethane (PU) film (1 mil thick) was obtained from American polyfilms (Branford, CT), EGF was kindly donated by Zyme Fast Inc. (Winnipeg, MB), Acrylic acid (AA), Benzophenone (BP), N,N'-methylenebisacrylamide (MBA), CH (MW = 70,000) were purchased from Sigma-Aldrich (Oakville, ON), Silicon wafers, Human dermal fibroblast cells (ATCC, PCS 201-010), Quantikine® human EGF ELISA kit were purchased from Cedarlane (Burlington, ON), MTS cell proliferation kit was purchased from Promega (Madison, WI), Azocoll, MMP-9 (Enzo life Sciences), Doxycycline hyclate (Sigma, M.W - 512.94 g/mol), Alexa fluor 488

Part 01: **Layer by layer assembly of EGF on PAA-PU film.**

The purpose of Part 01 is threefold: 1) to evaluate whether the small growth factor EGF can be deposited using the layer by layer system, 2) to assess whether the release of EGF can be controlled with its bioactivity retained, and 3) to assess whether the treatment results in accelerating cell migration and proliferation by using a simple wound model.

$(CH/EGF)_{20}$

Part 02: Layer by layer assembly of DOX + EGF on PAA-PU film.

The purpose of Part 02 is to add Doxycycline (DOX), a protease inhibitor on top of deposited EGF (Part 01) using a layer by layer assembly and then measure its ability to release both DOX and EGF in a controlled manner. Azocoll assay is used to evaluate the protease inhibition by doxycycline. The bioactivity of released EGF and the native EGF is compared. To evaluate the inhibition of proteases and the ability of EGF to accelerate healing, MMP-9 proteases are added to a simple wound model to mimic chronic wounds. As represented by following:

$(CH/DOX)_5 + (CH/EGF)_{20}$

PART 01: Layer by layer assembly of EGF on PAA-PU film

4.2 Surface modifications of films and silicon wafers

Surface modification was done on two different substrates - PU film and silicon wafer. The surface of the PU film was modified using the interpenetrating network technique (IPN) and the surface of the silicon wafers was modified using the plasma technique. In both cases, the surface was modified with polyacrylic acid (PAA) in order to generate a negatively-charged carboxylic acid group on the surface.

4.2.1 Immobilization of carboxylic acid groups onto PU films

The purpose of immobilization was to introduce anionic groups (carboxyl group), which act as anchoring groups for the deposition of EGF. The PU film was surface-modified with acrylic acid (AA) to form an interpenetrating network. [106] Briefly, a PU film was first swollen and treated in solution containing 20 ml of methanol, 4.118 ml of AA, 0.1338 mg of MBA (a cross linking agent) and 0.3009 mg of BP (a photo initiator) for 1 hour at 40^0 C. After 1 hour, the swollen film was exposed to UV irradiation (365 nm) for 2 hours to effect the formation of IPN. Finally, the un-reacted monomer and loosely attached polymer were removed by washing the film in water solution for 8 hours. The resultant PU film was referred to as "PAA-PU".

4.2.2 Immobilization of carboxylic acid groups onto silicon wafers

Silicon wafers were used as a model substrate for the study of LbL deposition (Characterization) of CH/EGF polyelectrolytes because as PU films cannot be used for characterizing.

4.2.2.1 Preparation of silicon wafers

Silicon wafers were washed using a soap solution by rubbing the surface of the wafer using a scrubber for a period of 10 to 15 minutes continuously. This was done in order to remove any minute dust particles on the surface of the wafer. Then the wafer was dried at 30 ^{0}C for 10 minutes under vacuum.

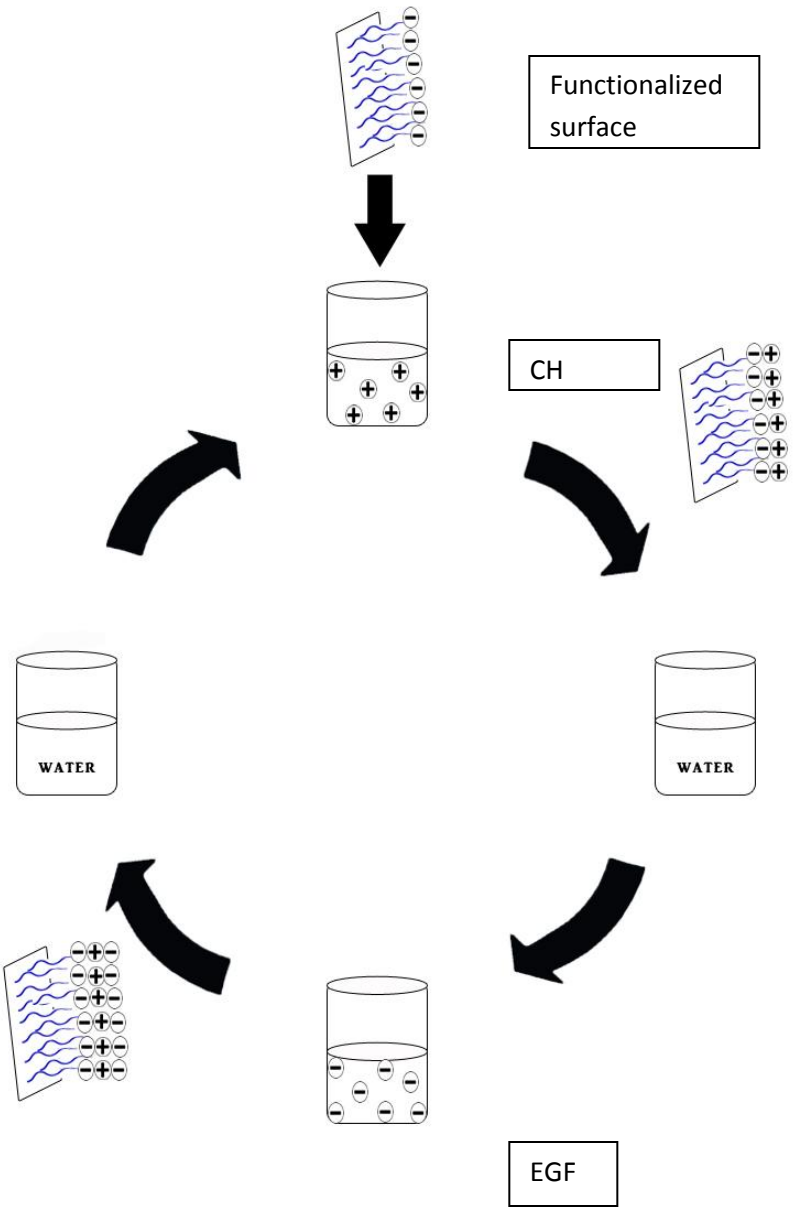
4.2.2.2 Immobilization of carboxylic acid groups

A 4 by 4 cms silicon wafer was coated with PAA by plasma polymerization in a USB 8 Plasma Activation & PECVD coating system (Plasma Technology, Reading, PA). The processing conditions were as follows: frequency - 13.56 MHz; chamber pressure - 0.3mbar; processing duration – 9 minutes. But before coating the surface of silicon wafer with PAA, the silicon wafer was quenched with oxygen for 20 minutes in order to activate the surface for the plasma deposition. Once the surface was quenched with oxygen, then PAA-PU was deposited on the wafer for 3 minutes.

4.3 LbL assembly of polyelectrolytes

To achieve LbL encapsulation of EGF, a cationic polyelectrolyte - CH was chosen to form an ion pair with EGF. CH and EGF dipping solutions were prepared by using ultrapure water with a resistivity of 18.2 MΩ.cm @ 25 °C from a Milli-Q plus (Bedford,

MA). A 3% acetic acid solution was prepared in which the CH was dissolved with a concentration of 1mg/ml at pH 4.2. Later, the pH of the CH was raised to 5.8 using 1M NaOH thereby, lowering the final concentration to 0.8mg/ml. The reason for raising the pH of the CH is that acidic conditions of CH may affect the bioactivity of EGF during successive layer depositions. EGF was dissolved in pH 7.4 (0.1M) phosphate buffer saline (PBS) solution at the concentration of 100 µg/mL. The PBS was also prepared using pure water. The amount of monobasic and dibasic was calculated based upon the molarity and the volume required. Both Silicon wafers and modified PU films were cut to the dimension of approximately 2 × 2 cms before LbL assembly of CH and EGF. The substrates were first dipped into CH solution and allowed to sit for 15 minutes. Afterwards, the substrates were rinsed 3 times in ultrapure water. Then the charge on the substrate was reversed by dipping them into EGF solution for 15 minutes and rinsed 3 times using ultrapure water. This procedure was repeated for a predetermined number of times depending on the experimental design. For the purpose of characterizing the growth of the layers, 20 layers $(CH/EGF)_{10}$ were deposited on the silicon wafer. After each layer was deposited, the silicon wafer was dried at 30 ^{0}C for 15 to 20 minutes under vacuum and was subjected for measurements. For the other *in-vitro* experimental designs, apart from characterization, the layers were deposited on the modified PAA-PU film and then dried at 30 ^{0}C for 30 minutes under vacuum but not intermittently.



Schematic Diagram 2: Illustration of layer-by-layer assembly of polyelectrolytes.

4.4 Characterization of the modified film and wafer

A colorimetric titration was used to quantify the carboxyl density on PAA-PU film. The growth of CH/EGFs on wafer was characterized using:

1. Contact angle measurements - the surface hydrophilicity was measured at each layer that was deposited.
2. Atomic force microscope (AFM) - the surface topography was analyzed at every 5 layers.
3. Ellipsometry - the thickness was measured at every 5 layers similar to AFM.

Before subjecting these wafers to measurements, the wafers were dried and all superficial dirt was removed using a nitrogen gun.

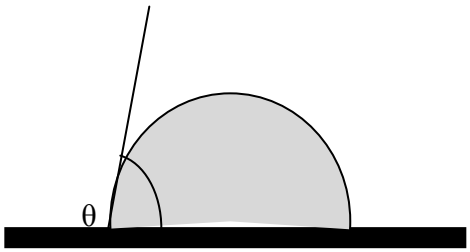
4.4.1 Colorimetric titration

In order to quantify the amount of carboxyl group on PAA-PU film, a colorimetric titration technique was employed. PU films which measured 2 by 2 cms were immersed in 5ml of ethanol solution containing thionine acetate dye (0.1mg/ml) and then shaken using wrist shaker machine for overnight at room temperature. After dyeing, the films were removed and washed with ethanol for 3 times until non-specifically adhered dye molecules were removed. [107] The absorbencies of the residue dye solutions and rinsing solutions were measured using UV-vis spectrophotometer at 605 nm wavelength and compared with the initial (standard) dye solution. The difference obtained was used to determine of the concentration of carboxyl group on PAA-PU film.

4.4.2 Contact angle measurement

The contact angle measurement was used to measure the hydrophilicity of the deposited layers. After one layer of polyelectrolyte was deposited, the wafer was rinsed with ultrapure water, dried at 30 ^{0}C for 10 to 15 minutes and then small size dust particles were removed using a nitrogen gun. The contact angle was measured by using a standard

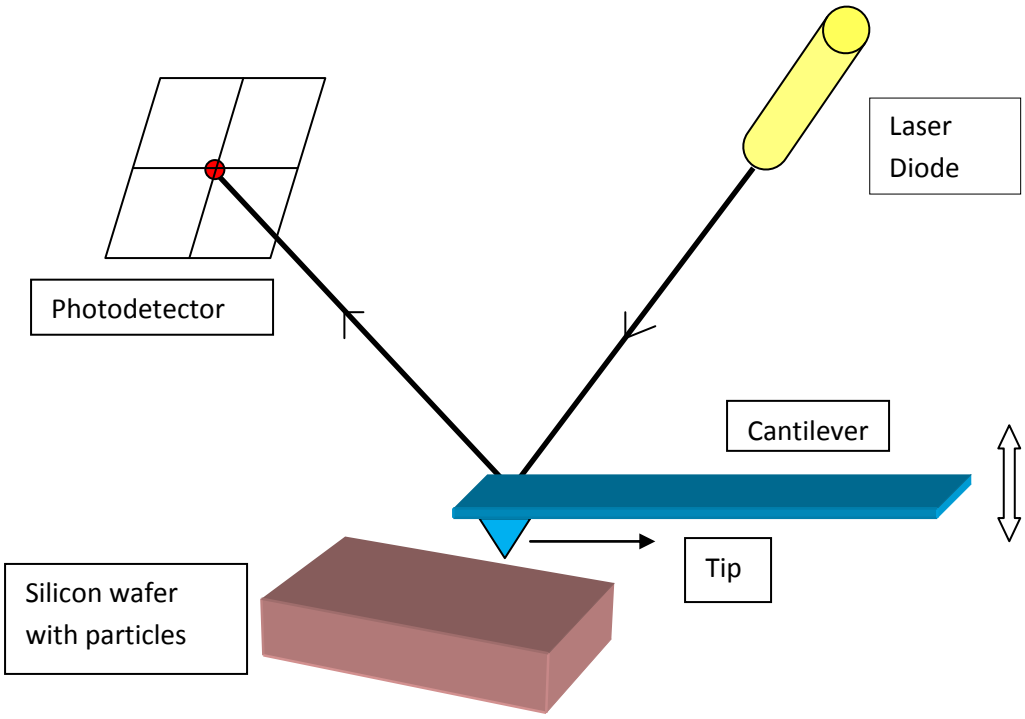
Rame-hart Model 200 goniometer. A 2 µL droplet was applied from the syringe onto the sample and the contact angle was determined at various spots on the wafer. (n=5)



Schematic Diagram 3: Mechanism of contact angle measurement.

4.4.3 AFM (Atomic force microscopy)

In order to investigate the surface topography of the deposited layers, AFM was used. Silicon wafer samples were examined using a Nanoscope III scanning probe microscope (Veeco, D3100) in tapping mode after purging the samples with a nitrogen gun. Nanoscope's commercially available n+ silicon cantilevers with a force constant of 10-130 N/m and a resonance frequency around 300 KHz were used for measuring the surface topography. The images were scanned through an area of 10 by 10 µm and then zoomed into 3 by 3 µm. The deposited wafer was scanned at various spots within 2 by 2 cms.

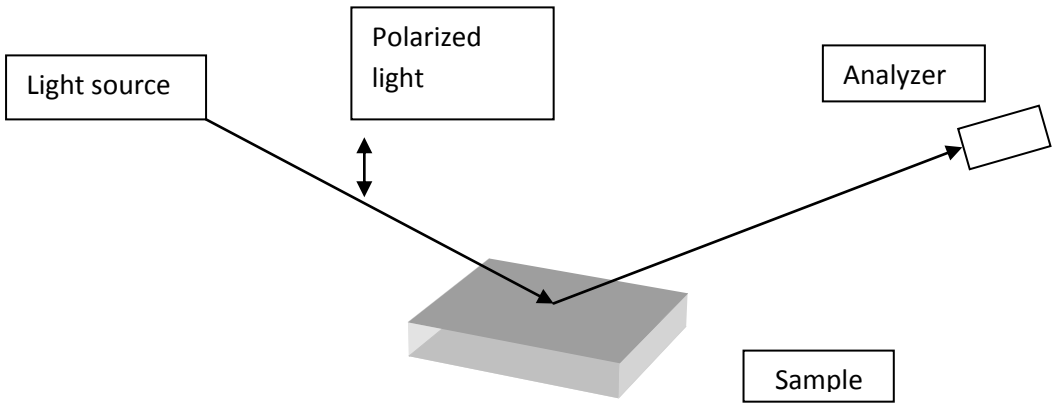


Schematic Diagram 4: Working principle of AFM

4.4.4 Ellipsometry

In order to determine the thickness of the deposited polyelectrolytes, ellipsometry measurements were performed using J.A. Woollam (Model M-2000D) at a wavelength of 500 nm and at angles of incidence of 55^0, 65^0, and 75^0. The polyelectrolyte layers were built on a silicon wafer. Before depositing the layers on the wafer, the wafer was cleaned

using soap solution and continuously rubbed with a scrubber for 5 minutes. Twenty layers $(CH/EGF)_{10}$ were built on the silicon wafer and the thickness was measured at every 5 layers. A model was developed as the thickness of the silicon wafer was constant. This model consisted of 2 Layers -- the first layer as silicon oxide whose value was a fixed parameter and then second layer was the deposited polyelectrolyte layers measured at intervals of 5 layers after 5, 10, 15 and 20 Layers deposition. On each sample, thickness was measured in 20 different positions. The overall thickness measurement was calculated as the average of all these 20 measurements.



Schematic Diagram 5: Mechanism of Ellipsometry

4.5 *In-Vitro* Release of EGF

CH/EGF was deposited on PAA-PU film and used to evaluate the release profile of EGF. 40 layers $(CH/EGF)_{20}$ were built on PAA-PU film. The film was stored at -20 ^{0}C until use. To release the drug, the film was immersed in 10 mL of PBS solution in a tightly sealed vial to prevent any evaporative loss of water. The drug was released in this

media in a thermostat water bath (37 ^{o}C). Two mL of the solution was removed from the above solution at predetermined time points and this two mL was replaced with two mL of fresh PBS solution. Predetermined points were chosen as representatives to estimate the concentration of the drug release. Release was done in PBS (pH 7.4) media inside a thermostat water bath (37 ^{0}C) for a period of seven days.

The concentration of the released EGF was determined by Enzyme linked immunosorbent assay (ELISA) using Human EGF Quantikine ELISA kit (R&D systems, Minneapolis, MN, USA). [108, 109] All the measurements were conducted in triplicate.

4.6 Bioactivity of released EGF

A cell proliferation test was used to evaluate the bioactivity of the released EGF in respect to the native EGF [110, 111]. Human neonatal fibroblast cells were cultured in fibroblast basal serum free media and 1% penicillin-streptomycin in a 37 ^{0}C, 5% CO_2 environment. 2×10^5 cells were seeded onto a 96 well plate in serum free media before released EGF samples were added. A MTS assay kit (CellTiter 96 Nonradioactive Cell proliferation assay, Promega, Madison, WI) was used to evaluate the bioactivity of released EGF. Once the cells were attached and confluent, both the released EGF and native EGF were added into each well and incubated for 24 hours. The native EGF was prepared at the same concentration of released EGF obtained at predetermined points of 8 hours, 1, 2, 3, 4, 5, 6, and 7 days. After 24 hours of incubation, 20µL of MTS/PMS complex was then added into these wells; cell proliferation was measured after 4 hours at 490 nm using an Opsys microplate reader. The released EGF was diluted 10 times in cell media before being added into the wells in order to minimize possible interferences of

PBS solution into which EGF was released. The percent of retained bioactivity of EGF was calculated as A/A_0, where A and A_0 were the absorbencies (490 nm) of cells cultured in the released and native EGF solutions, respectively (note: both solutions had the same concentration of EGF).

4.7 Actin Staining

Cells were grown to 80% confluence on collagen-coated glass cover slips. Three different treatments were used - (1) no serum, (2) regular media (Fibroblast basal medium), and (3) media with released growth factors. Cells were starved with any serum in all cases for 1 day prior to experiments. Cells in the third treatment group were incubated with released EGF at a concentration of around 800 pg/mL and incubated for 3 hours. Cells were then washed 5 times with PBS at pH 7.2 and fixed for 10 minutes using 4% para-formaldehyde (v/v) in PBS. Samples were washed 3 times with PBS and permeabilized in ice cold acetone for 3 to 5minutess. Finally the cells were labeled with Alexa Fluor 488 Phalloidin (Invitrogen), a stain which is specific for filamentous (F)-actin for 30 minutes at room temperature in dark. Cover slips were mounted on cleansed glass slides and viewed with fluorescence microscope (Zeiss Photo II) using neoflor objectives [111, 112]. Images were captured with a Dage camera and stored using Northern Eclipse software (Empix Imaging, Mississauga, ON). Gain, offset and exposure times were identical for all treatment groups.

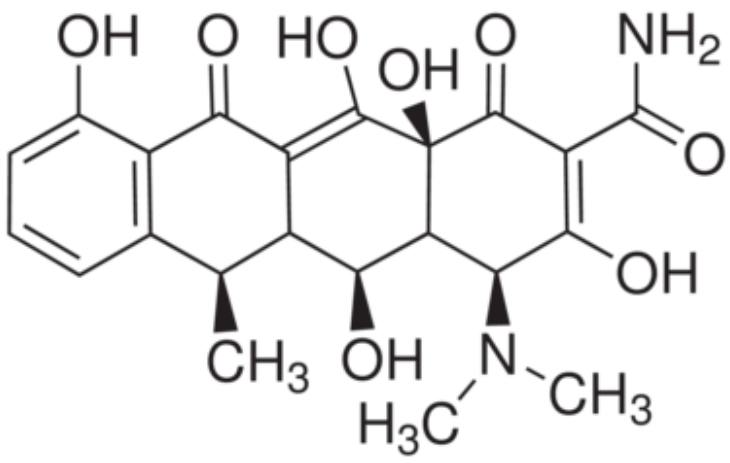
4.8 Wound model.

A simple skin model consisting of a layer of fibroblast cells was cultured with a seeding density of $3X10^5$ in 35mm round petri dishes coated with Type – I collagen and

incubated at 37^0C, 5% CO_2 until 90-95% confluence was reached. Two treatments were chosen to evaluate whether the released EGF can proliferate the cells at a higher rate when compared with no EGF released from control pristine PU film. The first treatment consisted of 40 layers of CH/EGF on PAA-PU film and in treatment two - pristine PU was used as control. For each dish, 2-3 scratches were made using 10-100µL pipette tip, resulting in well-defined scratch which did not induce any noticeable deformations in the polycarbonate substrate. Detached cells were then washed away using a cell culture media. Films were pre-wetted in the cell media and then media with film were added into these dishes. Cells growing on petri dishes were maintained in an enclosed heating stage at 37 ^{0}C and with 5% CO_2. [110] The well-defined scratch was photographed at 10X magnification every 5 minutes using a Zeiss Axiovert 200 inverted microscope until the wound was closed. Phase contrast images were captured using a 16X planapo objective lens and a Retiga Q-imaging camera. Optical calipers were used to measure wound closure rates using Northern Eclipse software.

PART 02: Layer by layer assembly of DOX + EGF on PAA-PU film.

In part 02, Doxycycline (DOX) - a broad spectrum antibiotic and a protease inhibitor of MMP-9 proteases was added on top of previously deposited EGF layers. DOX is even smaller than EGF and was really challenging to encapsulate using layer by layer assembly. Before depositing DOX on EGF, only DOX was added on top of the PAA-modified PU film to evaluate whether this small inhibitor DOX can be encapsulated using LbL system.



Schematic Diagram 6. Chemical Structure of Doxycycline Hyclate

Similar to part 01, Doxycycline was deposited on PAA-PU modified film for *in-vitro* experiments. For characterization, the doxycycline was deposited on the modified silicon wafer.

4.9 Immobilization of carboxylic acid groups onto PU films and silicon wafer

The process of immobilization of carboxylic group on PU films and silicon wafer was similar to the immobilization described in section 4.2.1 and 4.2.2.2 respectively.

4.10 LbL of polyelectrolytes (DOX, EGF and CH)

To achieve LbL encapsulation of DOX, previously used cationic polyelectrolyte - CH was chosen to form an ion pair with DOX. CH and DOX dipping solutions were prepared using ultrapure water with a resistivity of 18.2 MΩ.cm @ 25 °C from a Milli-Q plus (Bedford, MA). 3% acetic acid solution was prepared in which the CH was dissolved with a concentration of 1mg/ml at pH 4.2, later the pH of the CH was raised to 5.8 using 1M NaOH. DOX was dissolved in pH 6.8 (0.1M) Phosphate buffer saline (PBS) solution

at the concentration of 2mg/ml, 1mg/ml and 0.5mg/ml. The PBS was prepared using pure water.

The dimensions of the silicon wafer and film was 2 by 2 cms. The substrates were first dipped into CH solution and allowed to sit for 15 minutes. Afterwards, the substrates were rinsed 3 times in ultrapure water. Then the charge on the substrate was reversed by dipping them into DOX solution for 15 minutes and rinsed for 3 times using ultrapure water. This procedure was repeated for a predetermined number of times depending on the experimental design. For the purpose of characterizing the growth of the layers 20 layers $(CH/DOX)_{10}$ was deposited on the silicon wafer. After each layer was deposited the silicon wafer was dried at 30 ^{0}C for 15-20 minutes under vacuum and was subjected to measurements. For the other *in-vitro* experimental designs, apart from characterization, the layers were deposited on the modified PAA-PU film and then dried at 30 ^{0}C for 30 minutes under vacuum but not intermittently.

4.11 Characterization of the modified film and wafer

Similar to part 01, the characterization was done using contact angle measurements, AFM and Ellipsometry as described above.

4.12 In-Vitro Release of DOX

CH/DOX deposited on PAA-PU film was used to evaluate the release profile of DOX. Ten layers $(CH/DOX)_5$ were built on PAA-PU film and the film was stored at -4 ^{0}C until use. To release the drug, the ten layers deposited film with a size of 2 by 2 cms was immersed into ten ml of PBS solution in a tightly sealed vial to prevent any evaporative loss of buffer and the drug was released in this media in a thermostat water

bath (37 ^{o}C) at 70 rpm. Three mL of the solution was removed from the above solution at predetermined time points and this three mL was replaced with three mL of fresh PBS solution. Predetermined points were chosen as representatives to estimate the concentration of the drug release. Release was done in PBS (pH 7.4) media for a period of three days. The released DOX was detected using UV-vis spectrophotometer at 274nm. [100, 101]

Once DOX is tuned, then DOX is added on top of previously deposited EGF (Part-01) as both simultaneously released in ten ml of PBS solution in a tightly sealed vial. The released EGF is detected using ELISA and the DOX using UV-vis spectrophotometer. The bioactivity of released EGF is evaluated by MTS assay as discussed in section 4.6.

4.13 AZOCOLL assay

Azocoll is a protein dye conjugate, widely used to evaluate the protease inhibition test. Azocoll is basically an insoluble bright red Azo-dye to which ground collagen is attached. [113- 117.] Azocoll was first washed using phosphate buffer. 125mg of Azocoll was weighed and suspended in 25 ml of 50mM phosphate buffer pH 7.4. The Azocoll suspension was gently stirred using a magnetic stirrer for 2 hours at room temperature. After 2 hours, the mixture was centrifuged at 10,000 x g at room temperature for 10 minutes. The supernatant was removed and the process was repeated again. The washing of the Azocoll is done to remove any degraded peptide which may cause high background in protease activity assay. Two treatments were chosen (1) Released DOX from 5 bilayers of $(CH/DOX)_5$ PAA-PU film (2) In-situ contact which entailed exposing

the protease film containing five bilayers of $(CH/DOX)_5$. In the case of released DOX - 50µL of protease (Active MMP-9) and 50µL were mixed in with 900 µL of Azocoll suspension prepared in 50mM phosphate buffer. [118] The mixture was placed in a water bath at 37 ^{0} C for 2 hours, and the tubes were inverted at regular intervals. After 2 hours the solution was centrifuged at 10,000 rpm for 10 minutes. The supernatant was used to measure the absorbance at 520nm using a UV-vis spectrophotometer.

4.15 Wound model.

A simple skin model consisting of a layer of fibroblast cells was cultured with a seeding density of $3X10^5$ in 35mm round petri dishes coated with Type – I collagen and incubated at 37^0C, 5% CO_2 till 90-95% confluence was reached. Three treatments were chosen to evaluate whether the released DOX can inhibit the proteases, creating a safe environment for EGF to be released. The released EGF can enhance the migration and proliferation of the cells at a higher rate when compared with only EGF released from PAA-PU film and in comparison with control pristine PU film. The first treatment consisted of ten layers of $(CH/DOX)_5$ + forty layers of $(CH/EGF)_{20}$ on PAA-PU film and in treatment two only 40 layers of $EGF(CH/EGF)_{20}$ on PAA-PU film and in treatment three pristine PU was used as control. For each dish, 2 to 3 scratches were made using 10-100µL pipette tip, resulting in a well-defined scratch which did not induce any noticeable deformations in the polycarbonate substrate. Detached cells were then washed away using cell culture media. Films were pre-wetted in the cell media and then media with film were added into these dishes. 100ng/ml of proteases was added into the wound environment, which consisted of the film for analysis. Cells growing on coverslips were maintained in an enclosed heating stage at 37 ^{0}C and with 5% CO_2. [110] The well-defined

scratch was photographed at 10X magnification every 5 minutes using a Zeiss Axiovert 200 inverted microscope until the wound was closed. Phase contrast images were captured using a 16X planapo objective lens and a Retiga Q-imaging camera. Optical calipers were used to measure wound closure rates using Northern Eclipse software.

CHAPTER 5

Results and Discussions

Part 01: Layer by layer assembly of EGF on PAA-PU film

5.1 LbL assembly of CH/EGF bilayer on PAA modified PU film

To enable LbL assembly of EGF on PU film, positive or negative charges should be first introduced onto the surface. PAA (poly (acrylic acid)) was co-polymerized with a crosslinker MBA in the methanol swollen surface of the PU film to form an interpenetrating network (IPN) for the durable immobilization of PAA according previous reports published by our research group.[106, 119] FTIR spectra of treated and untreated PU films are presented in **Figure 2**. Unlike in the spectrum of untreated PU film (**Figure 2a**)where two peaks (1724 and 1700 cm^{-1}) had similar intensities, the 1700 cm^{-1} peak seemed to be spiked higher than 1724 cm^{-1} characteristic of carbamate bond in the spectrum of PAA-PU film (**Figure 2 (b)**). After subtraction, peak at 1695 cm^{-1} stood out indicating the presence of carboxylic acid group. The surface density of carboxylic acid group was quantified to be 330 $nmol/cm^2$ using a colorimetric titration method, described in section 4.4.1.

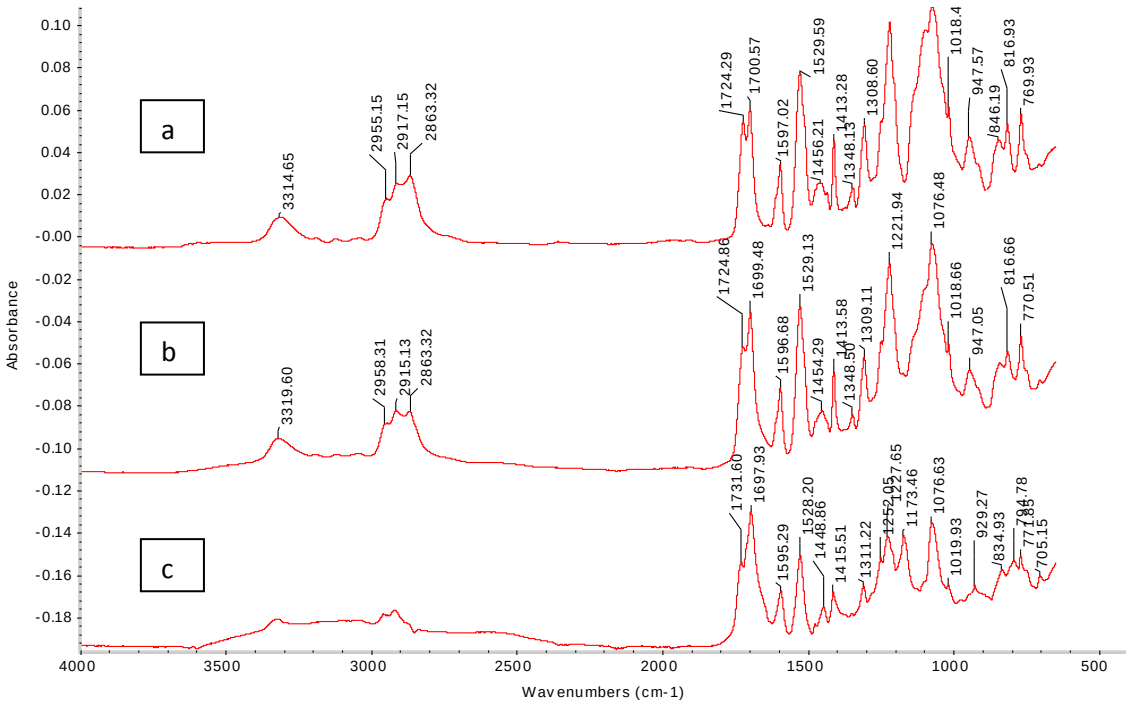


Figure 2. FTIR spectra of (a) untreated PU film; (b) PAA modified PU film; and (c) subtraction of (a) from (b)

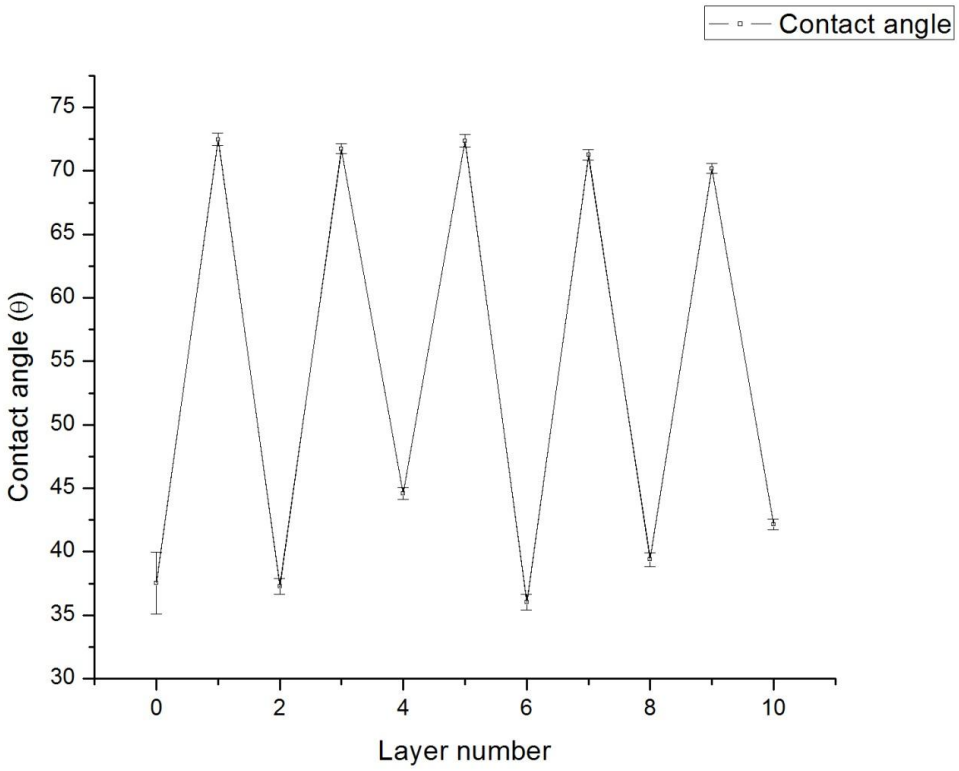
EGF is biologically stable in the pH range of 5-8 with maximum stability at pH 7.2.[120, 121] Therefore; it is more desirable to conduct the encapsulation of EGF at the biological pH (7.4) to maximize retention of its bioactivity. EGF is a weak polyelectrolyte whose charge and ionic strength highly depends on the pH. With an isoelectric point of 4.60, [121-123] EGF is negatively charged at pH 7.4. A polycation counterpart is required for the LbL assembly. It is known that polyelectrolyte complexes formed by protein and weak polyions could gradually dissociate in solutions with a pH close to the isoelectric point of one of the components. To allow the later release of encapsulated EGF, a weak cationic polyelectrolyte should be adopted. CH, a biodegradable polymer derived by deacetylation of the naturally occurring polysaccharide chitin, has been proven to promote the proliferation of cells and also

possess antimicrobial activity. Therefore, CH was chosen to serve as the polycation counterpart of EGF for the LbL assembly.

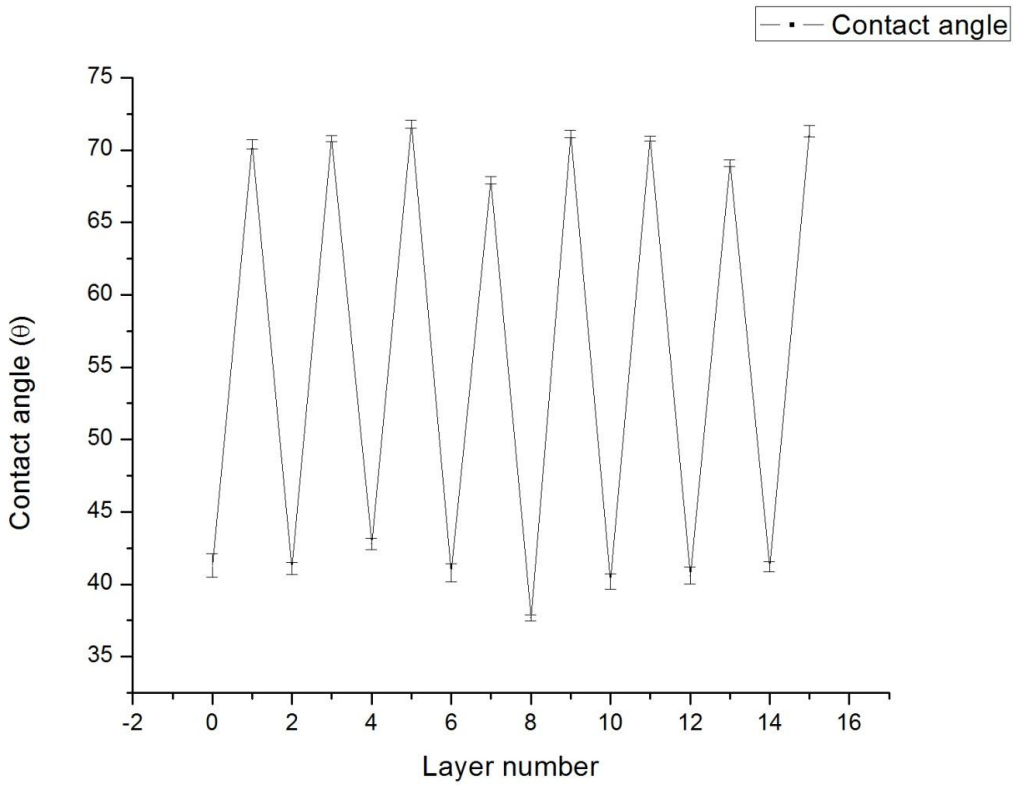
Surface charge density is a critical parameter for alternate adsorption of oppositely charged polyelectrolytes. CH has a high surface charge density at pH 4, but the bioactivity of EGF might be compromised at this pH. To better preserve the bioactivity of EGF, pH of CH deposition solution was raised to 5.8 at which the charge density of CH drops to 50-60% [124, 125] indicating that 50-60% of the CH molecules are still positively charged in the solution. This should be sufficient for the LbL assembly since it was reported that as little as 20% of charged blocks per chain could enable multilayer formation. [126]

5.1.2 Contact angle measurements

The contact angle test was adopted to monitor the CH/EGF film growth, i.e. the encapsulation of EGF. Contact angle measurements were conducted using a Rame-hart Model 200 goniometer (n=5). **Figure 3a** presents the contact angle measurement results of the PU film. The PAA-PU film became more hydrophilic with a lower contact angle (37.53±2.44) than the untreated PU film (97.12±1.67). An increase of contact angle to 73^{o} after the first dip coating in CH solution indicated a successful deposition of CH layer on the surface of PAA-PU film. The uniform coverage of CH was evidenced by the small variation of the measurement. A significant decrease of contact angle after the subsequent EGF assembly suggested the formation of a more hydrophilic EGF layer on the top. Alternate CH/EGF assembly was achieved up to ten layers as supported by the reciprocal change of contact angles (**Figure 3a**).


Contact angle
Contact angle (θ)
75
70
65
60
55
50
45
40
35
30
0
2
4
6
8
10
Layer number

(a)



(b)

Figure 3. a) Contact angle measurements of CH and EGF on silicon wafer (15 layers), b) contact angle measurements of CH and EGF on modified PU film (10 layers). In both images odd no's indicate the measurements for CH and even numbers represents EGF measurements.

5.1.3 LbL assembly of CH/EGF bilayer on PAA coated silicon wafer

In order to understand the topography of the CH/EGF bilayers and to estimate the thickness formed by these bilayers, a model substrate silicon wafer was used instead of PU film. Silicon wafer was coated with PAA by plasma polymerization. PAA was fed into a vacuum chamber and polymerized onto silicon wafer under the influence of plasma. Subsequently, CH/EGF bilayers were deposited following the same protocol. The

results of contact angle measurement (**Figure 3b**) lent support to the formation of the polyelectrolyte layer up to 15 layers.

5.1.4 AFM

AFM was employed to verify the deposition of the polyelectrolytes at regular intervals. The multilayers were scanned in tapping mode after the surfaces were gently blown with a stream of nitrogen. It can be seen from **Figure 4** that the surface roughness did not change significantly upon the deposition of PAA while the surface did become rougher upon the deposition of CH/ EGF. The root mean square (RMS) of the oscillation amplitude of AFM probe can be used as a quantitative measurement of surface roughness and is presented in **Table 2**. The RMS increased with the growth of polyelectrolyte film, from 0.355 nm silica wafer to 0.486 PAA coated silicon wafer to 2.20 nm for 5 polyelectrolyte layers, to 2.80 nm for 10 layers, to 3.02 nm for 15 layers. The rise in surface roughness after the deposition of 5 polyelectrolyte layers indicated the deposition of CH and EGF molecules. With further deposition, the RMS values increased to a lesser extent. One plausible reason is that further deposited polyelectrolytes cover the gaps formed in previous layers. Clearly the globular conformations or the peaks appeared as the LbL film was further constructed. Since the 'roughness' obtained from AFM did not correlate with the thickness of the polyelectrolyte film, ellipsometry was adopted to characterize the LbL film.

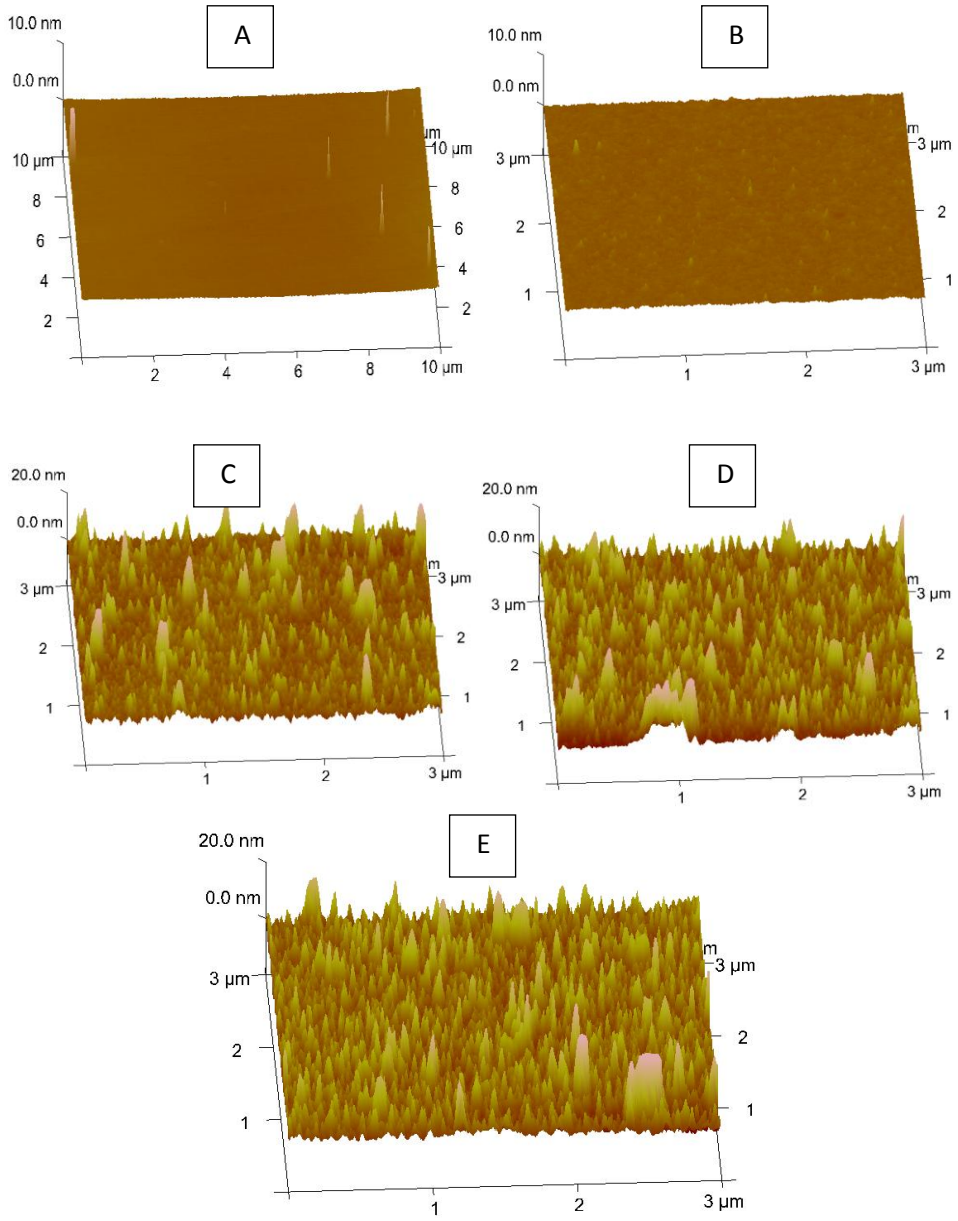


Figure 4. AFM images of a) silicon wafer b) modified silicon wafer (a layer of carboxyl group) c) 5 layers of CH/EGF d) 10 layers of CH/EGF e) 15 layers of CH/EGF.

Table 2. Surface roughness (RMS) values of deposited layers.

Parameters	Roughness (RMS) (nm)
Silicon wafer	0.355
Modified silicon wafer (Poly acrylic acid)	0.486
5 Layers of CH/EGF (CH top layer)	2.20
10 Layers of CH/EGF (EGF top layer)	2.80
15 Layers of CH/EGF (CH top layer)	3.02

As shown in **Figure 5,** the thickness of the CH/EGF film increased linearly with the number of layers with approximately 1.76 nm per bilayer. For big proteins such as lysozyme, the layer thickness of the protein can be as big as 4.5 nanometers. [127] As the hydrodynamic diameter of EGF is about 3.7 nanometers, [128] it is not surprising to find that CH/EGF bilayer thickness falls in the typical range of bilayer thickness of the linear polyions (1.5-2nm).[129] Steady increase of the thickness of CH/EGF bilayer suggests the lack of interlayer interpenetration of the polyelectrolyte in the deposition of ten layers of CH/EGF. To conclude, EGF could be effectively encapsulated on the –COOH functionalized surface with the counterion polyelectrolyte CH using the LbL deposition technique.

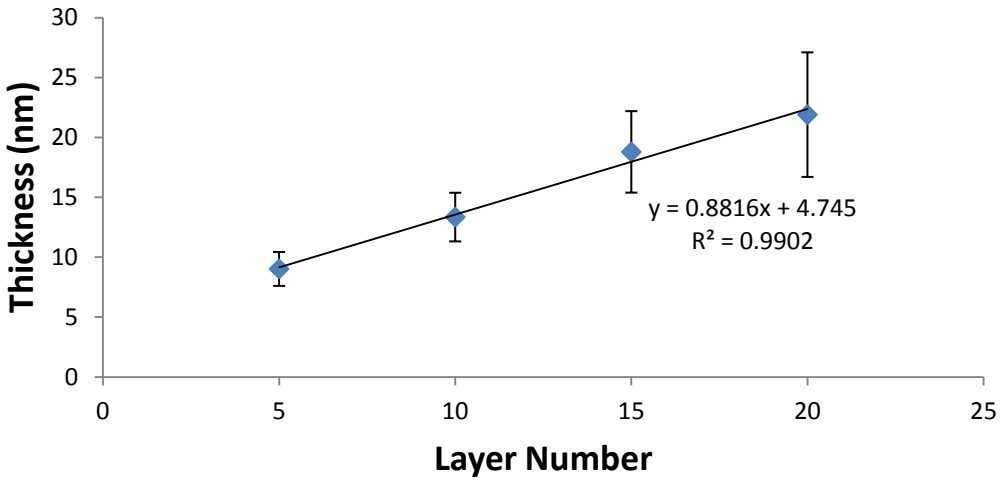


Figure 5. Thickness measurements of 20 layers of polyelectrolytes on silicon wafer.

5.1.5 *In vitro* EGF release from PU films

After the successful deposition of EGF, the release profile from the EGF containing PAA-PU film was studied. Firstly, 10 layers of polyelectrolytes i.e. $(CH/EGF)_5$ was constructed and released *in vitro*. Within one day, the released EGF only amounted to about 1.1 nanograms as shown in **Figure 6.** When 40 layers of CH and EGF were stacked onto PAA-PU film, a significant increase in the total dosage was found to be 22 nanograms. This means the amount of encapsulated and later released EGF is tunable depending on the number of fabricated polyelectrolyte bilayers. Approximately 76% of the EGF was released in the first 24 hours, which is advantageous since an initial higher dosage may help initiate cell responses in chronic wounds. The release was prolonged in a zero-order manner for three days and plateaued thereinafter with 99% EGF released on day six. The relatively high percentage of EGF in the first day is

attributable due to PU film swelling. As the film swells, small electrolytes such as phosphate can better diffuse into the CH/EGF bilayers to dissociate the electrostatic binding between CH and EGF, facilitating the release of EGF. Once the swollen film was saturated, the release rate diminished and was sustained for several more days. Based on experimental observations, the PAA-PU film could swell to approximately 2 times its original size in PBS solution in around 10 minutes. This observation was also supported by a zeta-potential measurement of the PAA-PU film in an adjustable gap cell with a SurPASS zeta-potentiometer. The gap between two pieces of PAA-PU film increased linearly with the swelling duration in the studied range: 0 to 30 minutes.

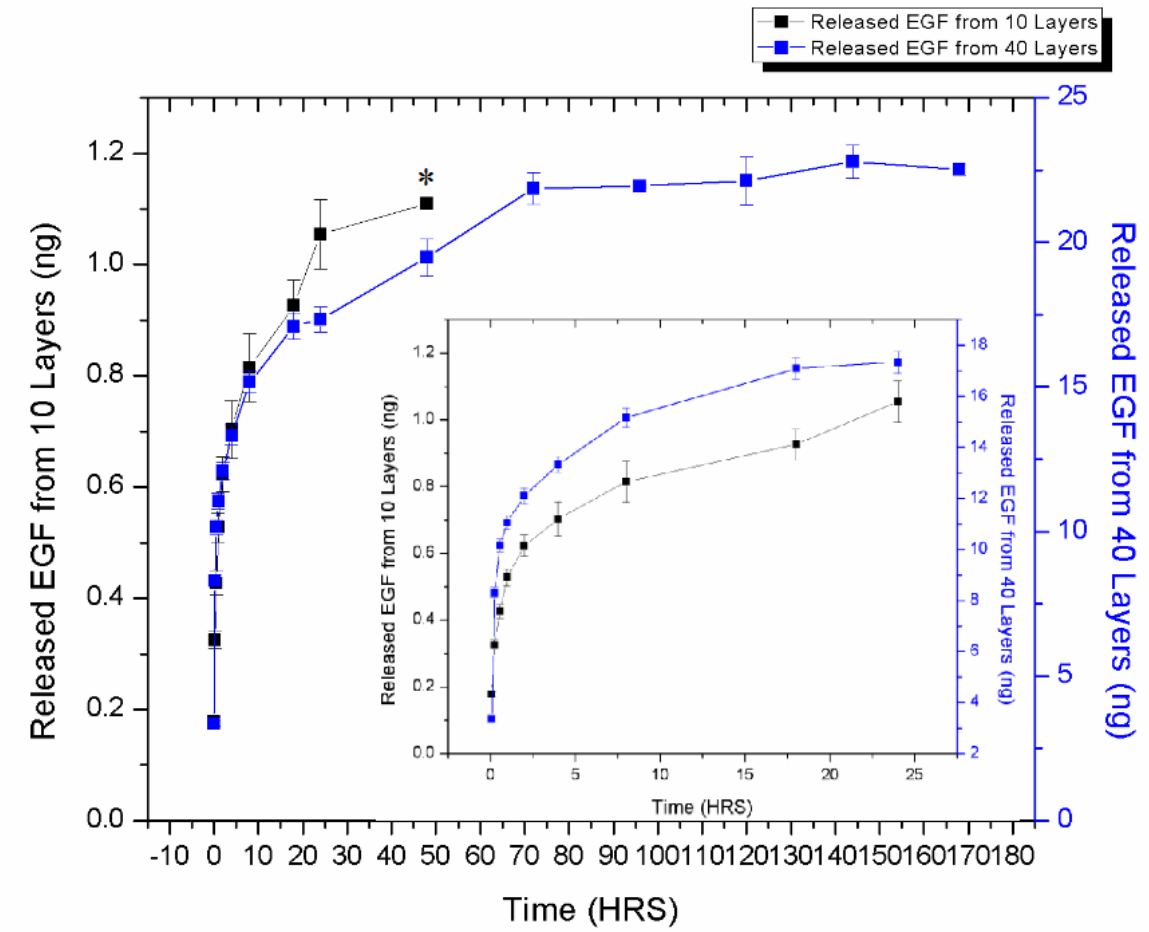


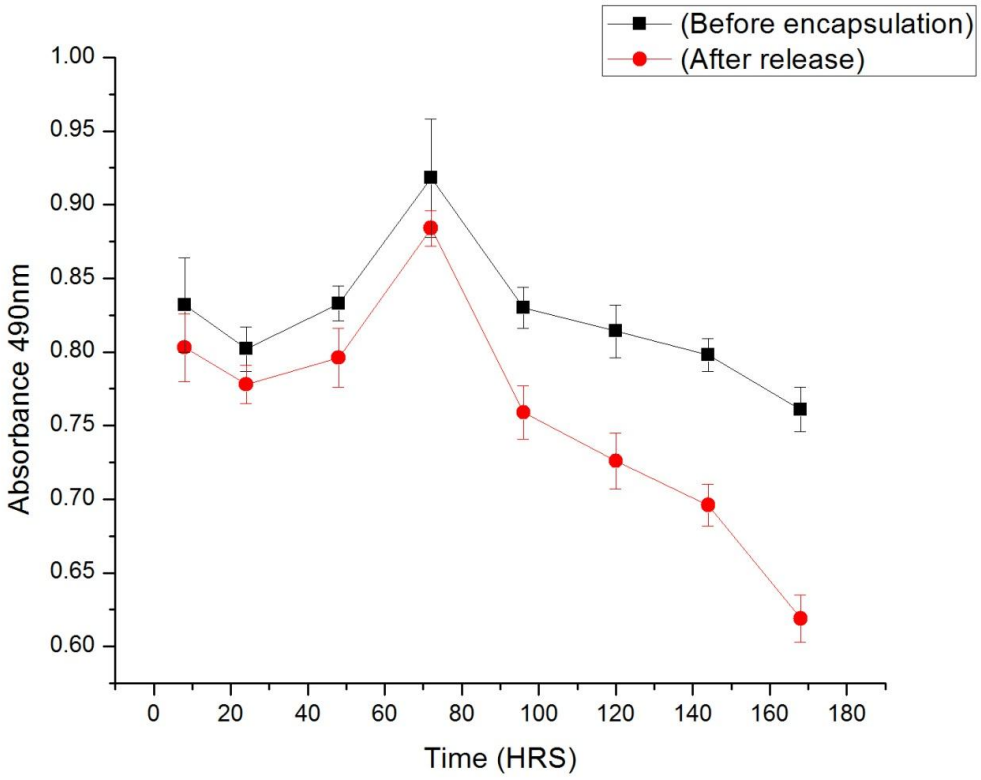
Figure 6. EGF release from PU films containing 40 layers and 10 layers. Inset showing the release profile up to 24hours. Quantification of the released EGF was done using ELISA assay. * indicates only a single point (n=1).

5.1.6 Bioactivity of Released EGF

We confirmed that EGF could be encapsulated together with CH onto the surface of the PAA-PU film using LbL technique and then be sustainably released. Another important question was whether the released EGF retained its biological activity. Researchers have applied the EGF topically and many others have released the EGF in a controlled manner. In one case, wounds on male Wistar albino rats healed much faster when they were covered with dextran hydrogels which are capable of releasing 61.6 µg EGF in 3 days. [130] In another case, EGF was released from pellets at a dosage as low as 4ng per day and still showed accelerated wound healing. [49], [55] Basic research of the percentage of retained bioactivity of released EGF should be conducted in comparison with native EGF to provide guidance for *in vivo* studies and even clinical trials.

In this study, the *in vitro* bioactivity of the released EGF was estimated using a cell proliferation assay. Neonatal fibroblast cells instead of keratinocyte cells were chosen, because the fibroblast cells are the 'first responders' in the wound healing process than keratinocytes. Furthermore, neonatal cells are more advantageous than the adult fibroblast cells since they are easy to grow. MTS assay was used to quantify number of proliferated cells. Released EGF was compared with the native EGF to obtain an evaluation of the percent of retained bioactivity by the encapsulated and released EGF. Released EGF was found to retain its bioactivity and induce proliferation of cells. **Figure 7** presents the absorbencies of the formazan reduced from MTS tetrazolium by the cells cultured with native and released EGFs, respectively. The released EGF was 96.9% bioactive after one day release and its bioactivity slightly reduced to 90% on the fifth day of release. Even after one week of release, the retained bioactivity was

still as high as 81%. This indicates that the bioactivity of EGF has not been compromised by the encapsulation technique which might be a feasible way for the delivery of bioactive EGF to the wound bed



Time (HOURS)	8	24	48	72	96	120	144	168
% Bioactivity	96.5	97	95.5	96.3	91.7	89.2	87.2	81.4

Figure 7. MTS cell proliferation assay quantifying the percentage of bioactivity retained by released EGF.

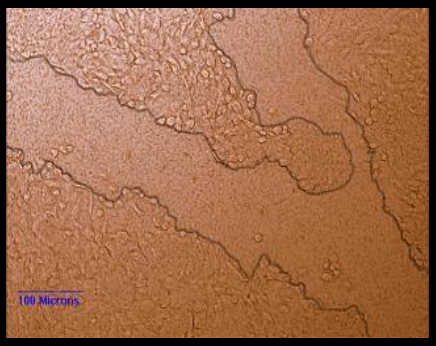
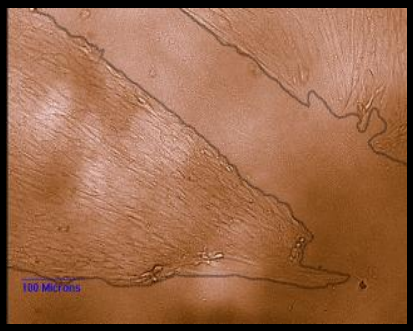
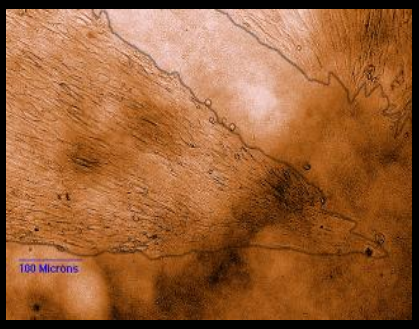
5.1.7 Wound model

A wound healing model was constructed to evaluate the healing promotion ability of EGF-loaded PU film. Human dermal fibroblast cells were seeded onto the collagen-coated petri dish; every 3 days once the cell culture media was changed. Once 90% confluence was achieved, an incision was made in the cell layer using a pipette tip. The EGF-loaded PU film and the control film were then laid above the cells containing media. Wound closure was measured under the microscope over a time period till the wound was completely closed.

The wound sample treated with EGF-deposited film stimulated migration as shown in **Figure 8**. The initial distance of the incision was around 120 µm in EGF-treated samples. The images from **Figure 8** indicate that fibroblast cells proliferated much faster in response to EGF when compared to the control where the movement was indeed slow. The cells were stimulated and migrated from one end to another as the EGF was released. In the case of Guinea pig tracheal epithelial cells (GPTEC), the addition of EGF (2.5-15 ng/ml) in wounds created by a rubber stylet showed the closure in 12 to 24 hours [131], which is in parallel to our results where the wound get closed almost in 20 hours when EGF was released from the LbL system.

The graphs in **Figure 9** show that the cells proliferated at a very constant rate in case of EGF. In case of the EGF containing film, fibroblast cells migrated at an average rate of 4.6 µm per hour whereas in the control sample (untreated PU film); fibroblast cells migrated at an average rate of 1.4 µm per hour. The slow migration was likely due to the fact that in the absence of EGF, the cells lacked stimulation. After 20 hours

treatment with EGF, the wound was almost closed while the control which took 70 hours to close. In summary, nanograms of EGF are sufficient to stimulate cells to migrate and proliferate so to close the wound in the in vitro model.

Wound model with released EGF	Time	Wound model without EGF (Control)
	**Start (0 Hours)**	
	**6 Hours**	
	**12 Hours**	

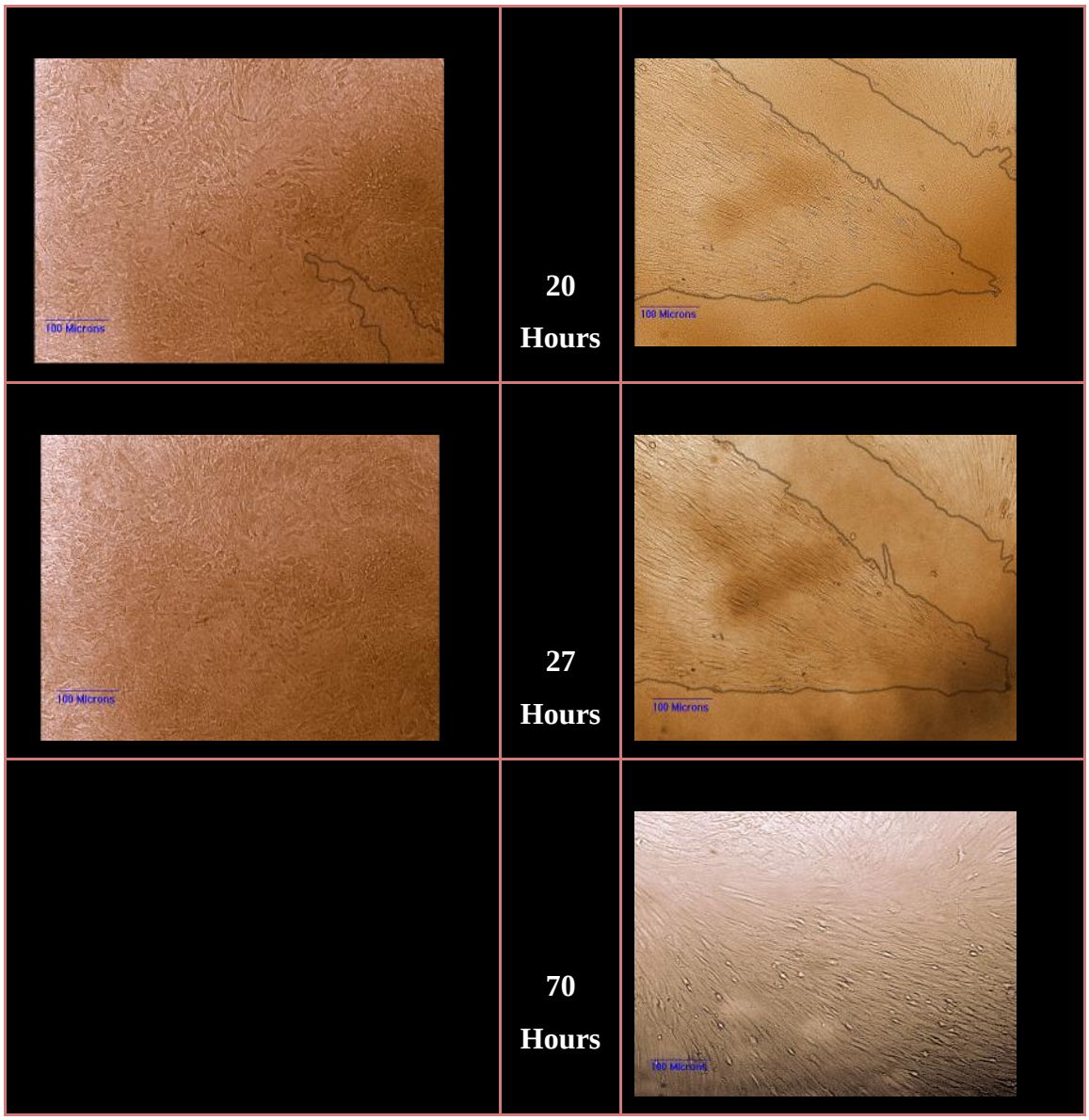


Figure 8. Healing of a scratched wound model covered with EGF deposited PU film and control - pristine PU film.

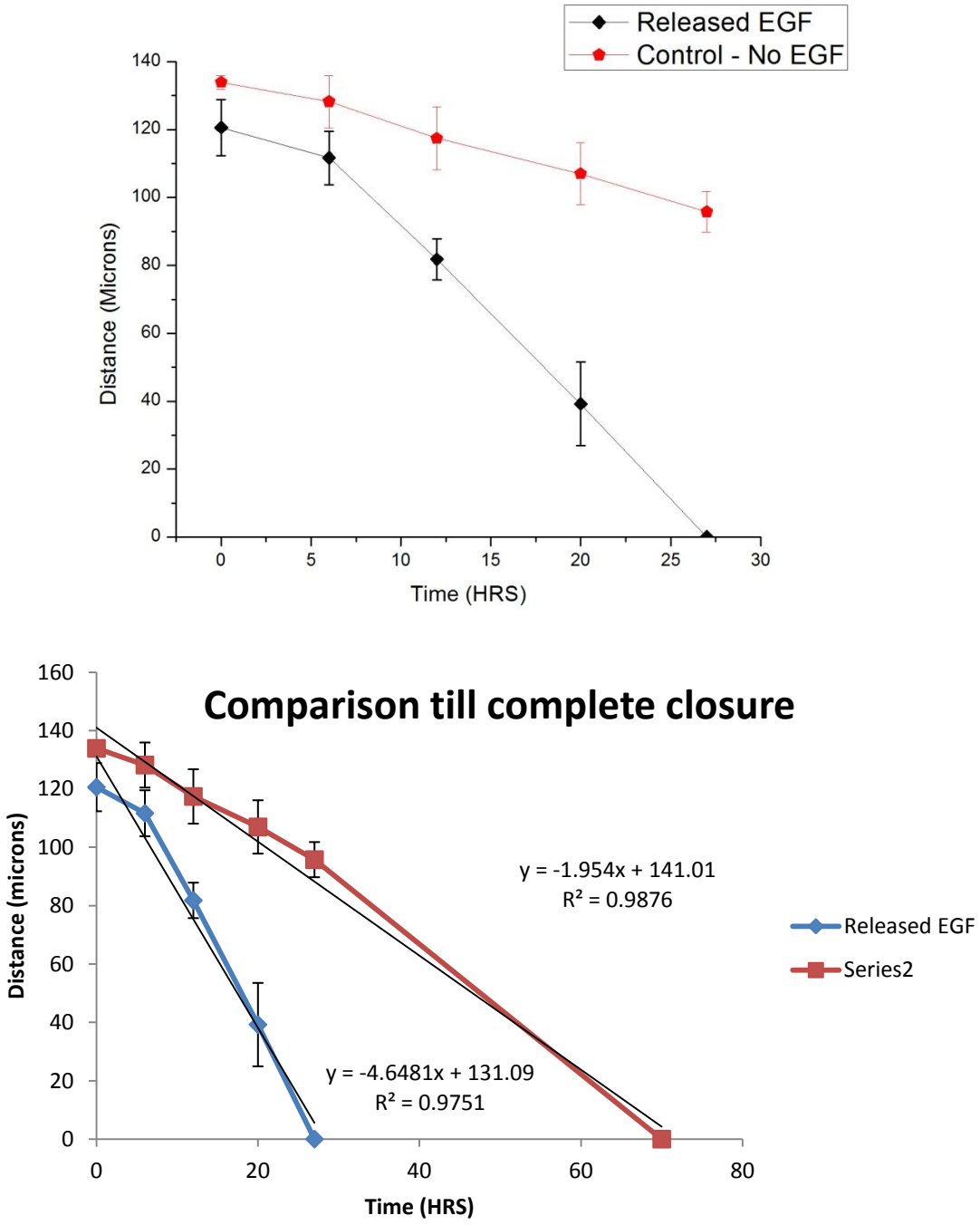
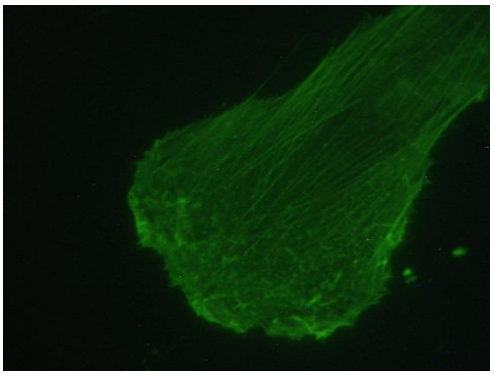
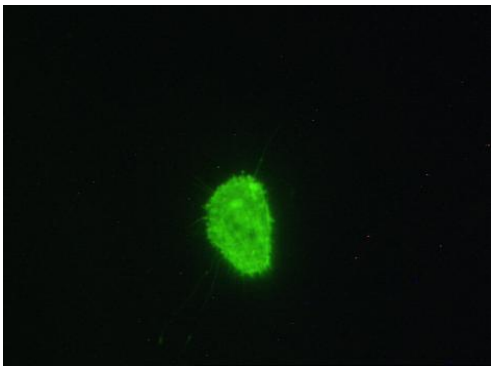
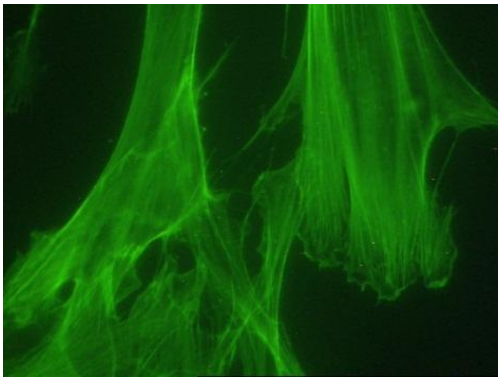
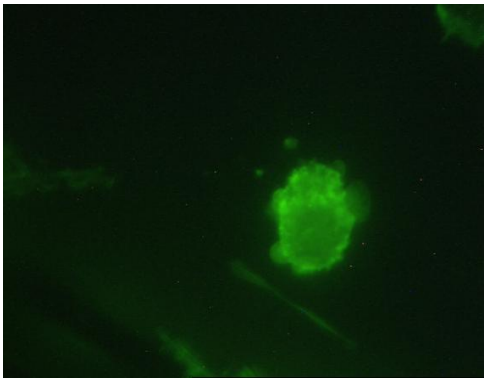
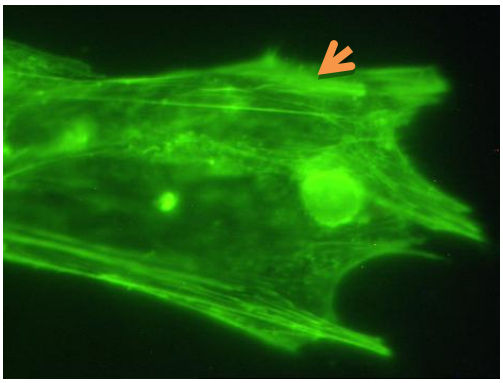
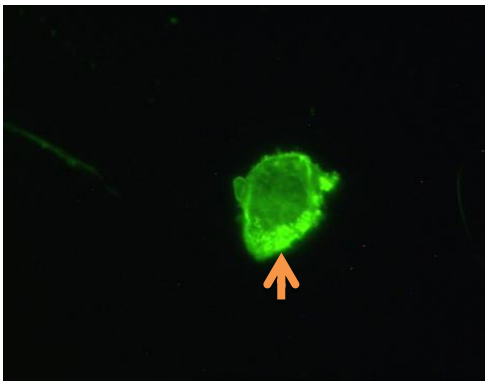


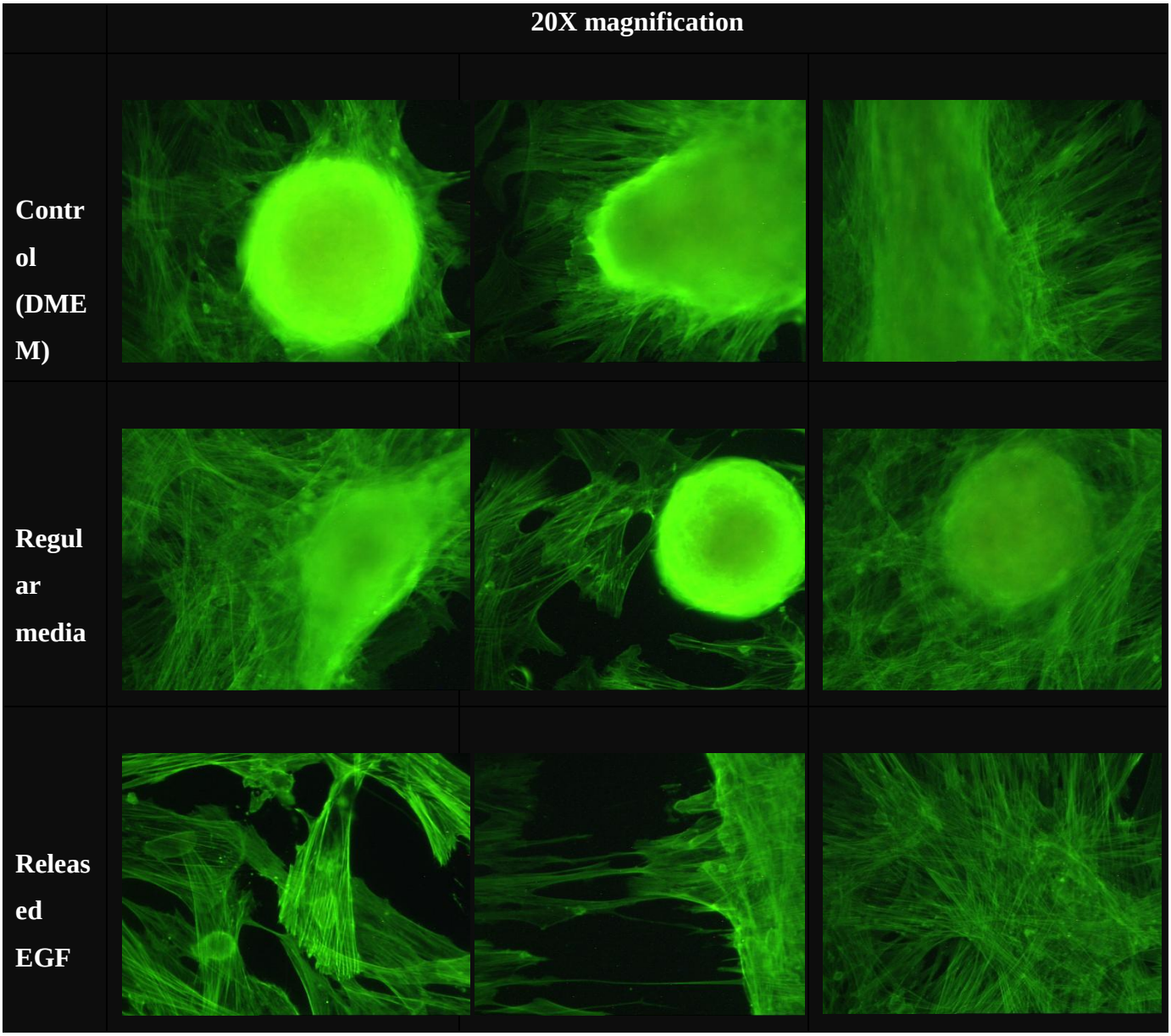
Figure 9. Wound gap as a function of healing duration. a) Indicating the cell migration rate for both released EGF and from Control till complete closure in case of released EGF, b) Cell migration rate till complete closure of control.

5.1.8 Actin staining of the fibroblast cells

F-actin staining was performed to localize actin filaments using Alexa fluor - 488 phalloidin dye. In **Figure 10a**, the control samples maintained with serum exhibited a marked reduction in F-actin compared to either EGF-treated or serum-treated cells. Cells exposed to released EGF showed a visible increase in actin filament density compared to the other treatments, which was especially pronounced in the leading edge of polarized cells and in areas of membrane ruffling shown by arrows in the **Figure 10a**. Additionally, F-actin staining highlighted cellular 'aggregates' or clumps of fibroblasts (**Figure 10b**) which were more prominent in serum-free and medium-exposed cells, but were markedly reduced in cells incubated in released EGF.

	40X magnification	20X magnification
Control (DMEM)		
Regular media		
Released EGF		

(a)



(b)

Figure 10. Immunostaining of actin filaments. a) Differences in F-actin density and distribution b) Formation of cellular aggregates.

Part 02: Layer by layer assembly of DOX + EGF on PAA-PU film

It is clearly evident from part-01 that the small growth factor EGF could be easily encapsulated and released with compromising its bioactivity. In second part, the challenge was to encapsulate doxycycline, which has very small molecules, with EGF, and release both the encapsulated drug into the wound bed. To respond to this challenge, we deposited only doxycycline with CH as counterion on PAA-PU modified film and on silicon wafer for characterization similar to Part-01.

5.2.1 LbL assembly of CH/DOX bilayer on PAA modified PU film

Similar to the process documented in Part 01, the surface of the PU film was modified using the IPN technique described in section 5.1. Doxycycline has an iso-electric point of 5 - 5.5. [132-134] At pH 6.8 it is negatively charged and can form an ionic pair with polycation CH.

5.2.2 Contact angle measurements

The growth of nano-layers (CH/DOX) on the wafer was analyzed by its surface hydrophilicity at each layer, which was measured by using Rame-hart Model 200 goniometer (n=5). Since the same modified wafer was used as in Part 01 was used in this experiment, the contact angle of the PAA layer was 41.96 ^{0}. As shown in **Figure 11**, layer 0 is representative of PAA indicating 41.3 ^{0}. After the first layer (CH) was deposited onto the modified silicon wafer, the contact angle was increased to 70.9 ^{0}. The uniform coverage of CH was evidenced by the small variation of the measurement. Once the second layer (DOX) was deposited the surface hydrophilicity increased from 70.9 ^{0} to 26.43^0 suggesting that a layer of DOX was deposited on the top of CH layer. Successful

deposition of alternating CH/ DOX assembly was achieved up to 20 layers as shown in **Figure 11**.

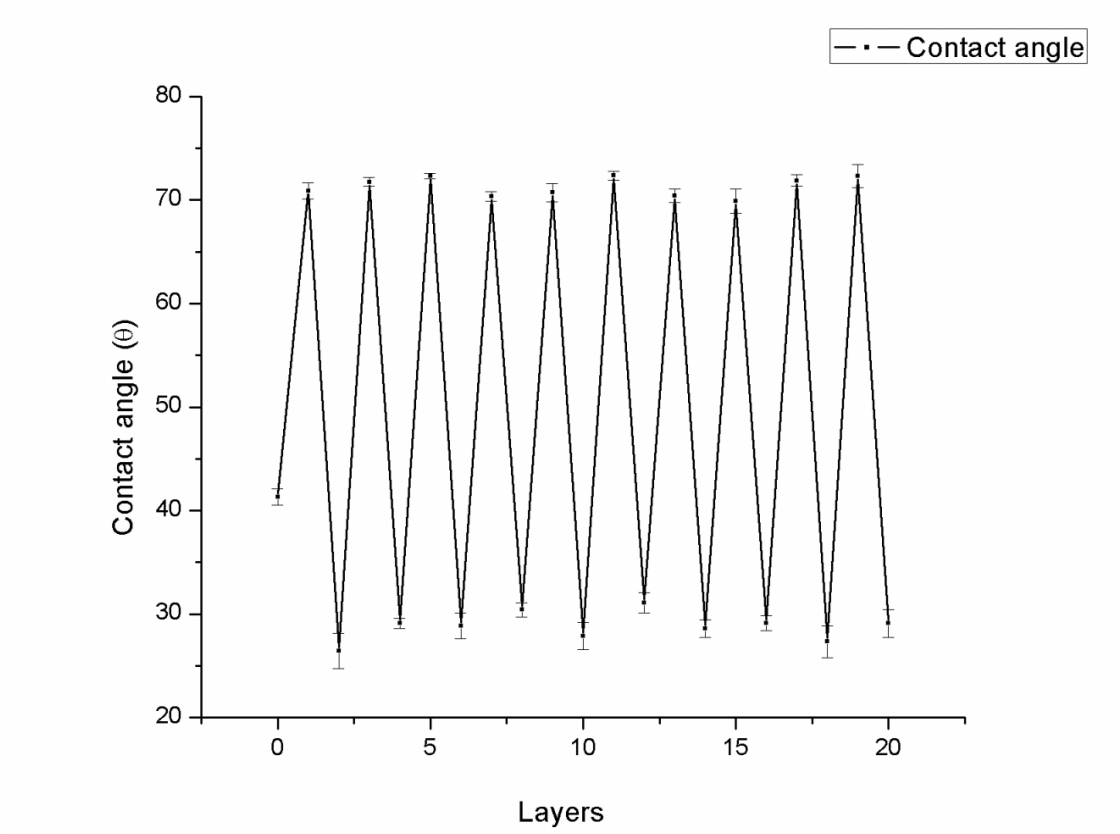


Figure 11. Contact angle measurements for CH and DOX on silicon wafer (20 Layers).

5.2.3 AFM

AFM indicated the change in the surface topography as the layers was deposited. Scanning was done after 5 layers were deposited; it was scanned in tapping mode after removing the superficial dust with a stream of nitrogen. **Figure 12** shows that the surface became rougher upon the deposition of CH and DOX. The RMS value increased as the layers were built in as shown in **Table 3.**

To evaluate the variation in the thickness of the layers, two treatments were administered by changing the pH of CH. In the first treatment, CH was deposited at pH 4.2, where most of the CH molecules were positively charged. In the second treatment, CH was deposited at pH 5.8 where only 40-50% of the CH was positively charged. The change in the pH of the CH changes the rate of deposition and the thickness of the layers. The surface roughness RMS of CH at pH 4.2 increased from 1.79 nm for 10 layers, to 3.32 nm for 20 layers. At pH 5.8 the surface roughness increased from 2.08 nm for 10 layers to 3.53 nm for 20 layers. These results show that CH deposited at pH 5.8 yielded a rougher surface than CH at pH 4.2. The increase in the surface roughness of CH at pH 5.8 is due to the formation of clumps rather than an even layer in case of CH at pH 4.2. With further deposition, the slope of RMS increase became less steep. One plausible reason is that further deposited polyelectrolyte's cover the gaps formed in preceding layers. Clearly globular conformations or peaks appeared as the LbL film was further constructed. However, the roughness parameter obtained does not provide any information about the thickness of the deposited layers; hence ellipsometry was used to determine the thickness of the layers.

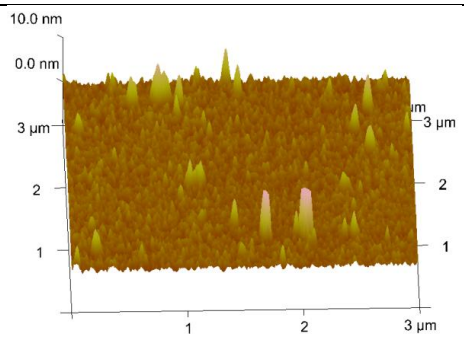
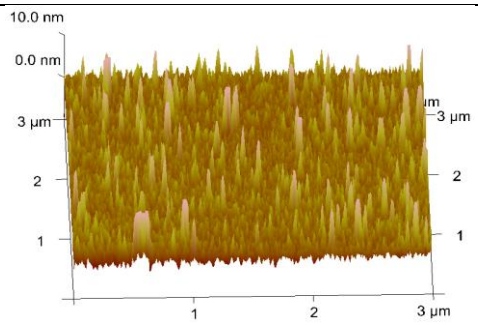
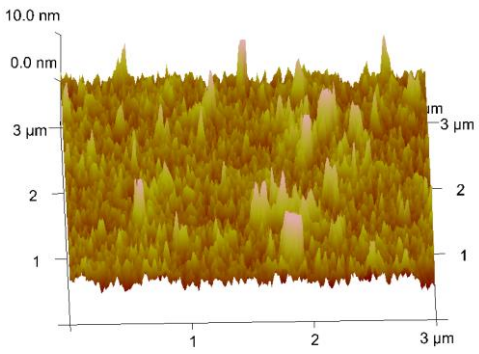
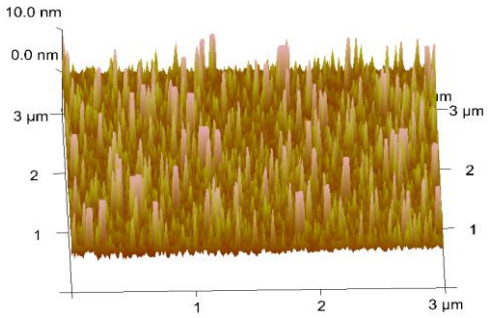
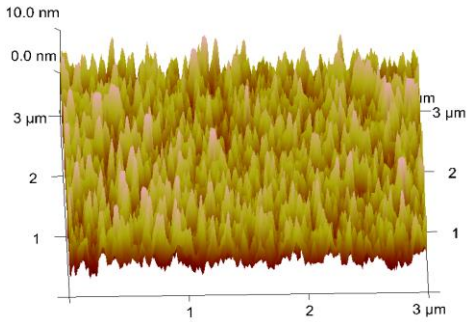
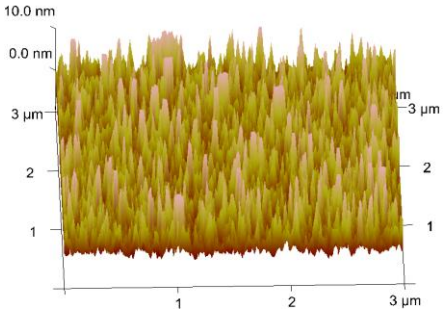
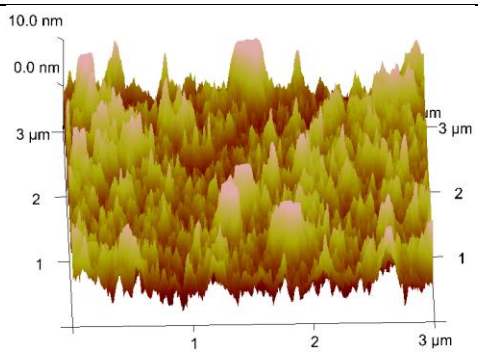
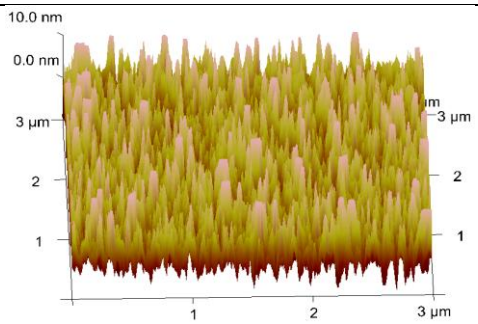
No. of Layers	CH at pH 4.2	CH at pH 5.8
5		
10		
15		
20		

Figure 12: Comparison of AFM images analyzed at CH pH 4.2 and pH 5.8.

Table 3. Comparison of surface roughness at CH at pH 4.2 and pH 5.8.

Parameters	**Roughness (RMS) (nm)** **CH at pH 4.2**	**Roughness (RMS) (nm)** **CH at pH 5.8**
5 Layers of CH/DOX (CH top layer)	0.909	1.51
10 Layers of CH/DOX (DOX top layer)	1.79	2.08
15 Layers of CH/DOX (CH top layer)	2.85	2.59
20 Layers of CH/DOX (DOX top layer)	3.32	3.53

Similar to AFM, two treatments were administered and analyzed for thickness using ellipsometry at different pH levels of CH as shown in **Figure 13.** We can clearly see the increase in the linearity of the growth of the polyelectrolytes on the silicon wafer with approximately 1.15 nm per layer in case of CH deposited at pH 5.8 and 1.69 nm per layer in case CH deposited at pH 4.2. This clearly indicates that at pH 4.2 the total thickness of 20 layers has increased when compared to thickness measured at pH 5.8. However, the total thickness in the case of CH deposited at pH 4.2 is 1.47 times thicker than CH deposited at pH 5.8. After 20 layers of deposition the thickness was 35.5 nm at

pH 4.2 when compared to 23.6 nm at pH 5.8. These results support the hypothesis that CH at pH 5.8 carries only 50-60% positive charge when compared to CH at pH 4.2. Hence, the rate of deposition is influenced highly by the surface charge density of CH. As the pH increases towards the isoelectric point of CH, the rate of deposition decreases due to the decrease in the charge density. Despite the smaller size of DOX, the increase in the layer thickness when compared to the deposition of EGF is due to the higher concentration of DOX used during deposition.

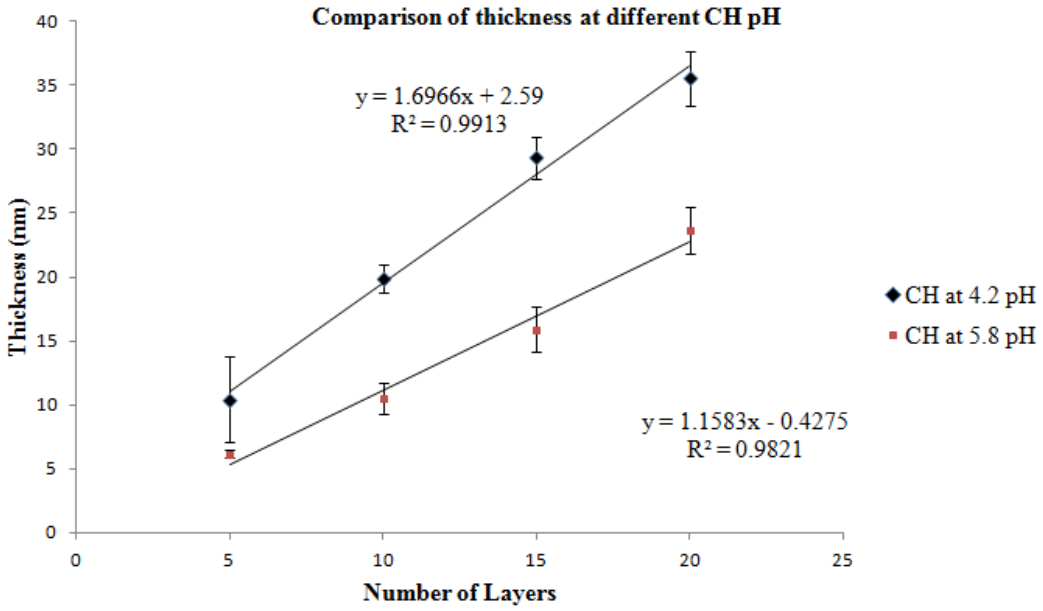


Figure 13. Thickness measurements for $(CH/DOX)_{10}$ CH at pH 4.2 and pH 5.8.

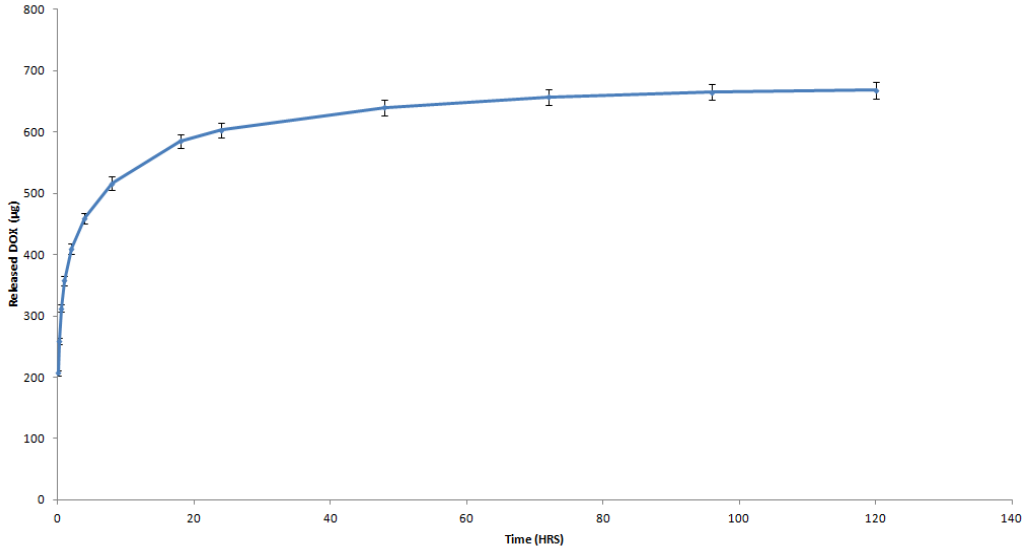
5.2.4 In Vitro release of EGF and DOX from PU films

The above characterizing data suggest that DOX can be easily encapsulated on the modified surface. In this section, results of experiments to ascertain the *in vitro* release are reported.

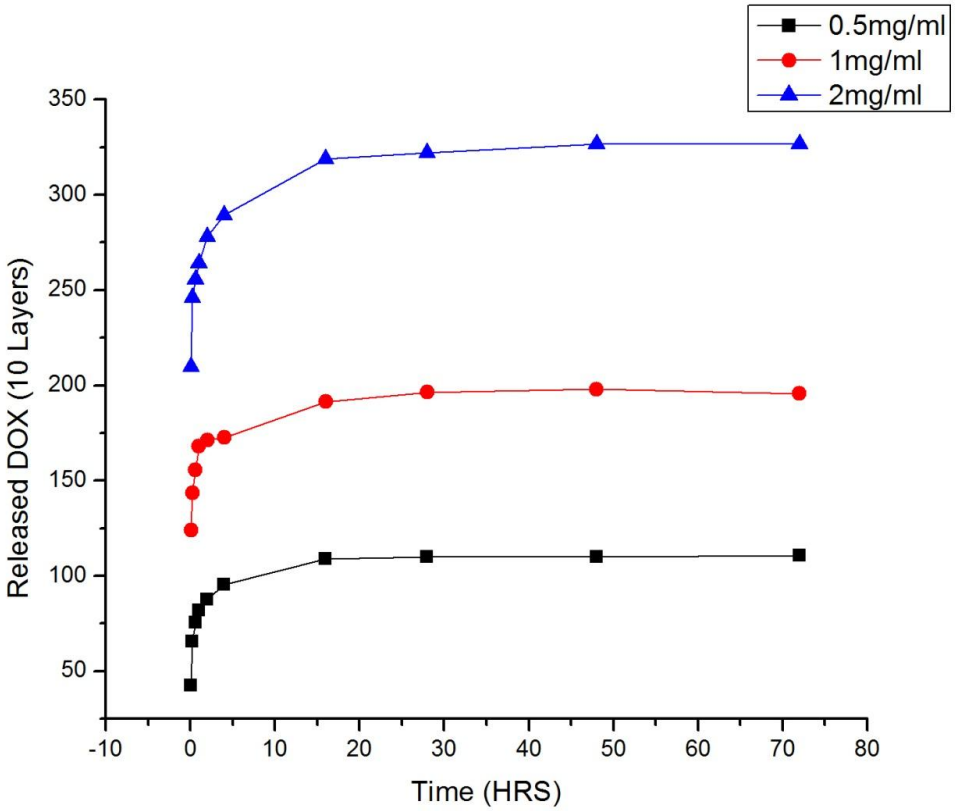
First, 10 layers of polyelectrolytes $(CH/DOX)_5$ were constructed and released in vitro for 5 days. **Figure 14a** shows that 603µg of DOX was released on the first day. After 2 days, 639 µg of DOX was released; after 5 days 668 µg of DOX was released. According to results reported by the other researchers, the amount of DOX released was excessive and could decrease cell viability. According to Shanmuganathan. S et al. the pure form of DOX reduces the viability of human fibroblast cells when applied more than 200 µg. But when the DOX is encapsulated and released from CH microspheres, it did not induce toxicity to cells. [135] Karna et al. showed that DOX applied at a concentration of 200µg/ml inhibited the proteases and did not have any effect on the viability of human skin fibroblast cells. However, when DOX was applied with a concentration of 500µg/ml, it decreased the viability of human skin fibroblast cells by 25%. [136] In this research, the amounts of DOX released were over 668 µg, which was 3 times the effective amount reported by Karna et al. and Shanmuganathan et al. Hence the amount of DOX released has to be reduced so that it does not affect the cell viability or its migration.

To reduce the amount of DOX released, in addition to the earlier prepared concentration of 2mg/ml, two concentrations of DOX were administered. The concentrations of the DOX - 0.5mg/ml, 1mg/ml at pH 6.8 were deposited on the PAA-PU film. Ten Layers of $(CH/DOX)_5$ were assembled and released in PBS pH 7.4, 37 ^{0}C in

water bath at 70 rpm for 3 days. **Figure 14b** exhibits the tunability of the amount of DOX released depending upon the concentration used.



(a)



(b)

Figure 14. Cumulative release of DOX from 10 layers with different concentrations of DOX a) 2mg/ml over a period of 5 days, b) 0.5, 1 and 2mg/ml over a period of 3 days.

From the above experiments, it is concluded that DOX can be encapsulated and easily released from the PAA-PU film with the provision of tuning the amount of DOX released. Now 5 layers of DOX $(CH/DOX)_5$ was deposited on top of 20 layers of EGF $(CH/EGF)_{20}$ totally adding upto 50 layers $(CH/DOX)_5$ + $(CH/EGF)_{20}$. Both DOX and EGF were released in 10 ml of PBS solution at 37 ^{0}C in a water bath at 70 rpm. This was

done in order to evaluate the effect of DOX on the release profile of EGF, whether EGF can be released before DOX is released.

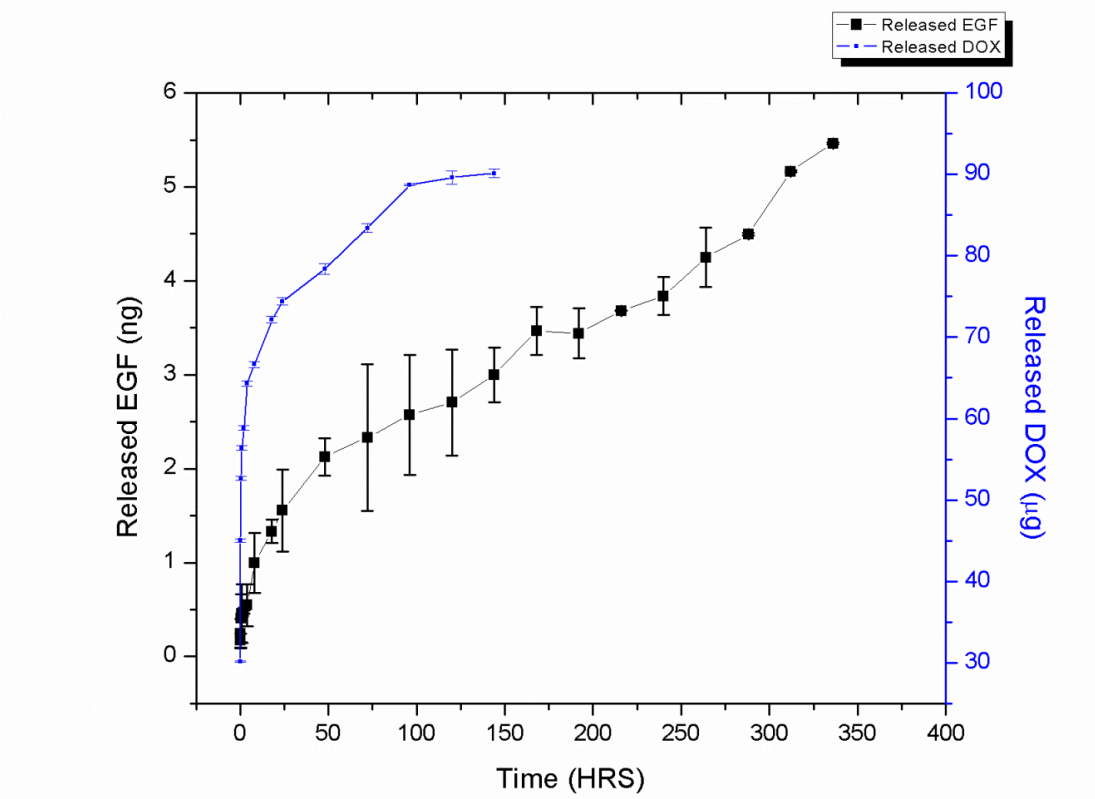


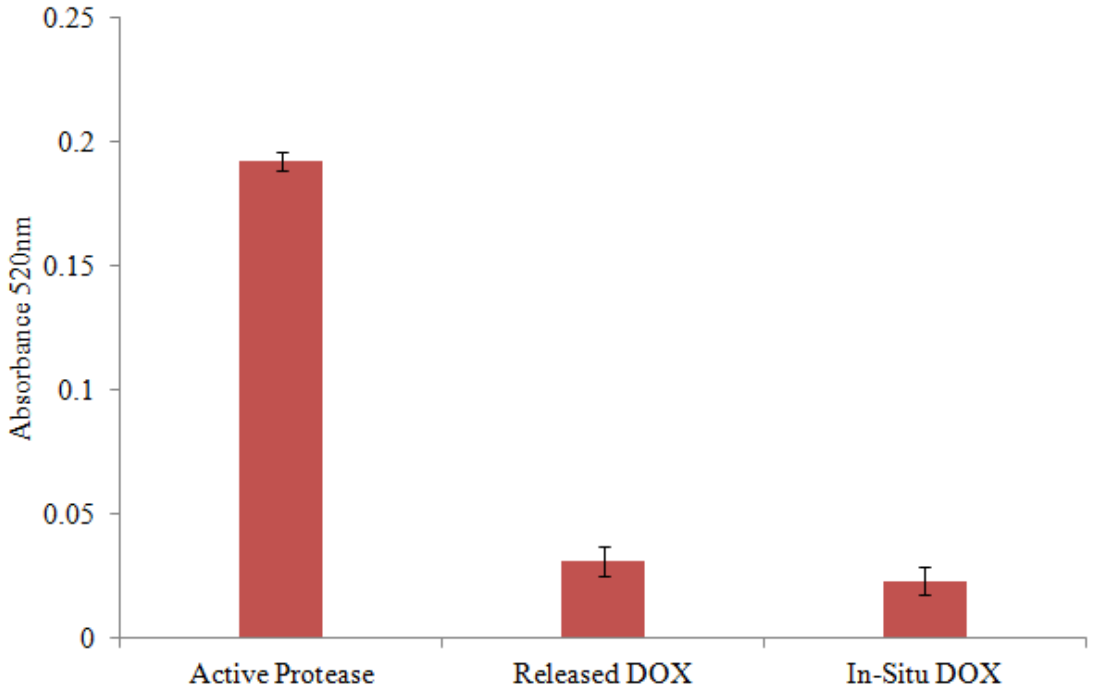
Figure 14 (c). Cumulative release of EGF from $(CH/DOX)_5$ + $(CH/EGF)_{20}$ from modified film.

As shown in **Figure 14c**, the amount of EGF released was affected by the addition of DOX. The released EGF was detected by ELISA assay similar to the previous Part 01 described in section 4.5. After 14 days only 5.5 nanograms of EGF was released. In Part 01, 22 nanograms of EGF released from 40 layers. This is due to swelling of the modified PU film. In earlier experiment when only EGF was deposited on a PAA-PU film with 40 layers of polyelectrolytes, the film swelled up to 18 hours and approximately 76% of the EGF was released in the first 24 hours. However, in this experiment with 50 layers of

$(CH/DOX)_5$ + $(CH/EGF)_{20}$, as the film swells for 18 hours, only DOX was released **(Figure 14c).** After the film reached its saturation point of swelling, only EGF is released in a more linear fashion. These results show that the initial swelling of the film facilitated the release of the topmost layers of the film. Once the swelling saturates, there is no further swelling to expel the EGF molecules. These results indicate that addition of DOX on top of EGF affects the amount of EGF released, but still 5.5ng of EGF was released, as detected by ELISA.

5.2.5 Evaluation of suppression of MMP-9 Proteases by DOX using AZOCOLL Assay

Azocoll assay is a quantitative method of evaluating the suppression of proteases by DOX. Dye is released from the matrix as the protease dissolves the collagen. Therefore, if the protease is not active, no color would be induced in the solution. **Figure 15a** shows the inhibition of active MMP-9 by DOX released from PAA-PU film and in-situ DOX.



	Active MMP-9		
	Protease	Released DOX	In-Situ Dox
Avg Value	0.1922	0.031	0.023
Std dev	0.003	0.006	0.0055

Figure 15 (a). Azocoll assay of selected samples.

Dye is released from the matrix as the protease dissolves collagen – higher absorbance indicates lower protease inhibition activity by the sample. **Figure 15a** and **15b** show that after 2 hours of incubation, both released DOX, and in-situ DOX significantly inhibited the proteases. This is due to the fact that DOX inhibited the proteases by metal binding thereby avoiding the degradation of collagen. In the case of

active proteases, the highly active proteases degraded collagen making the Azo dye soluble which develops color and produces higher absorbance.

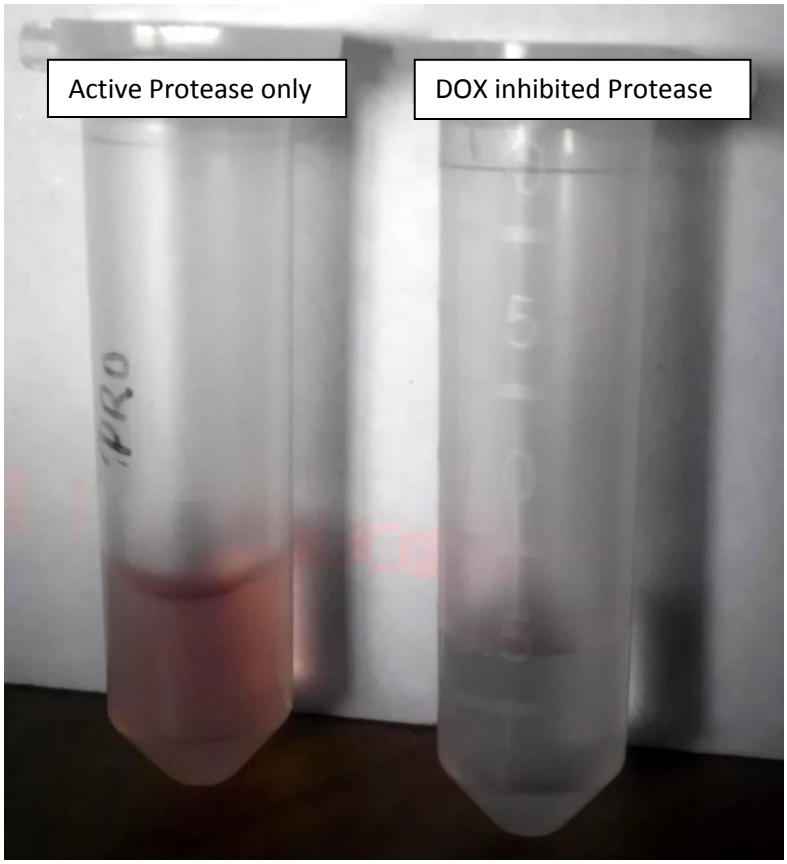


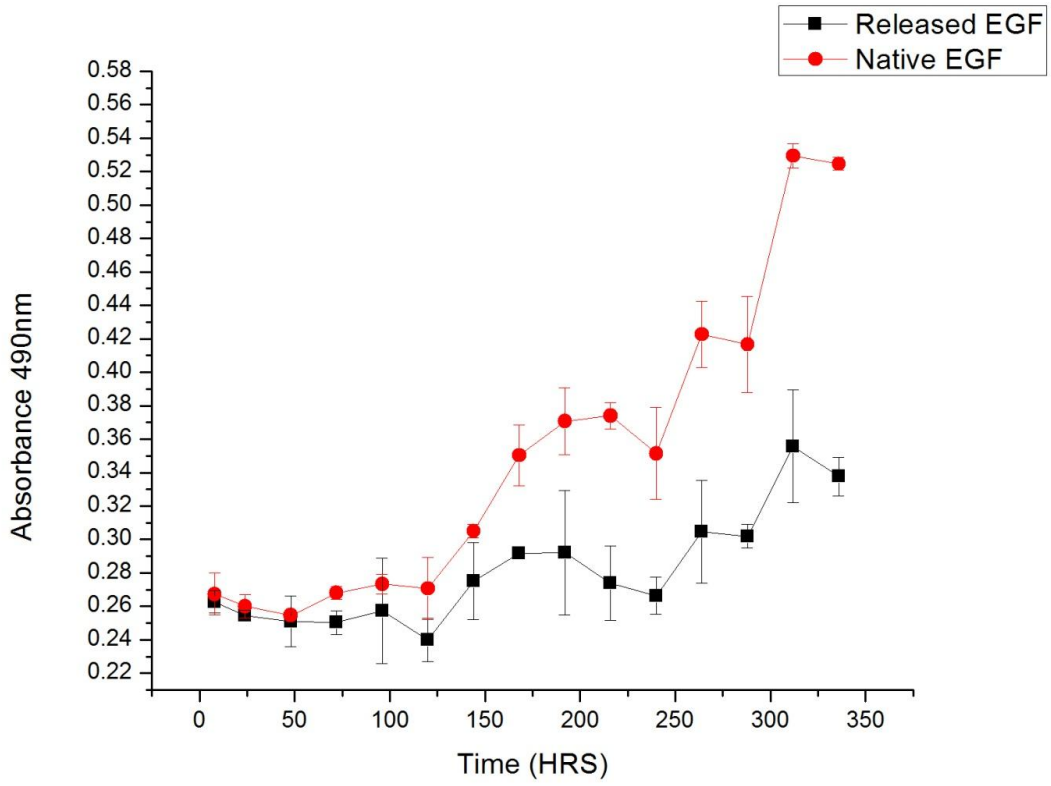
Figure 15 (b). Samples indicating the color developed by Active protease and protease inhibition by DOX.

5.2.6 Evaluation of Bioactivity of EGF released with DOX

In the previous section 5.1.6, it is clearly evident that released EGF was highly bioactive, inducing a mitogenic effect in the cells. The addition of DOX on the top of EGF to inhibit MMP-9 protease may threaten the bioactivity of EGF. An experiment involving cell proliferation assay was conducted to evaluate whether EGF retains its bioactivity or whether DOX compromised its bioactivity. The released EGF was

compared with native EGF to evaluate retained bioactivity by the encapsulated and released EGF.

Figure 16 shows that the released EGF was 97.9% bioactive after one-day release. After seven days, the bioactivity of the EGF dropped slightly to 83.25%. After fourteen days of release, the bioactivity of EGF was dropped to 64.35%. These results suggest that since the released EGF retained its bioactivity over a 14-day period, DOX did not affect the bioactivity of EGF during the encapsulation technique; nor did the encapsulation technique have any effect on the activity of EGF.



Time (HOURS)	8	24	48	72	96	120	144	168
% Bioactivity	98.2	97.9	98.5	93.4	94.1	88.6	90.1	83.2

Time (HOURS)	192	216	240	264	288	312	336
% Bioactivity	78.7	73.2	75.8	72.0	72.4	67.1	64.3

Figure 16. MTS cell proliferation assay quantifying the percentage of bioactivity retained by released EGF in presence of released DOX.

5.2.7 Evaluation of Healing Rate Using a Wound Model

Similar to part 01, a wound model was constructed with the purpose of evaluating the inhibition of proteases and the healing ability of EGF released from film.

Human dermal fibroblast cells were seeded onto the collagen-coated petri dish and once media was changed once three days. Once 90% confluence was achieved, an incision was made in the cell layer using a pipette tip. In order to mimic the amount of MMP-9 proteases observed in chronic wounds, 200µl of 100ng/ml MMP-9 was added to the wound system. Three treatments were administered in evaluating the healing performance.

(i) DOX + EGF loaded PU film

(ii) EGF loaded PU film only

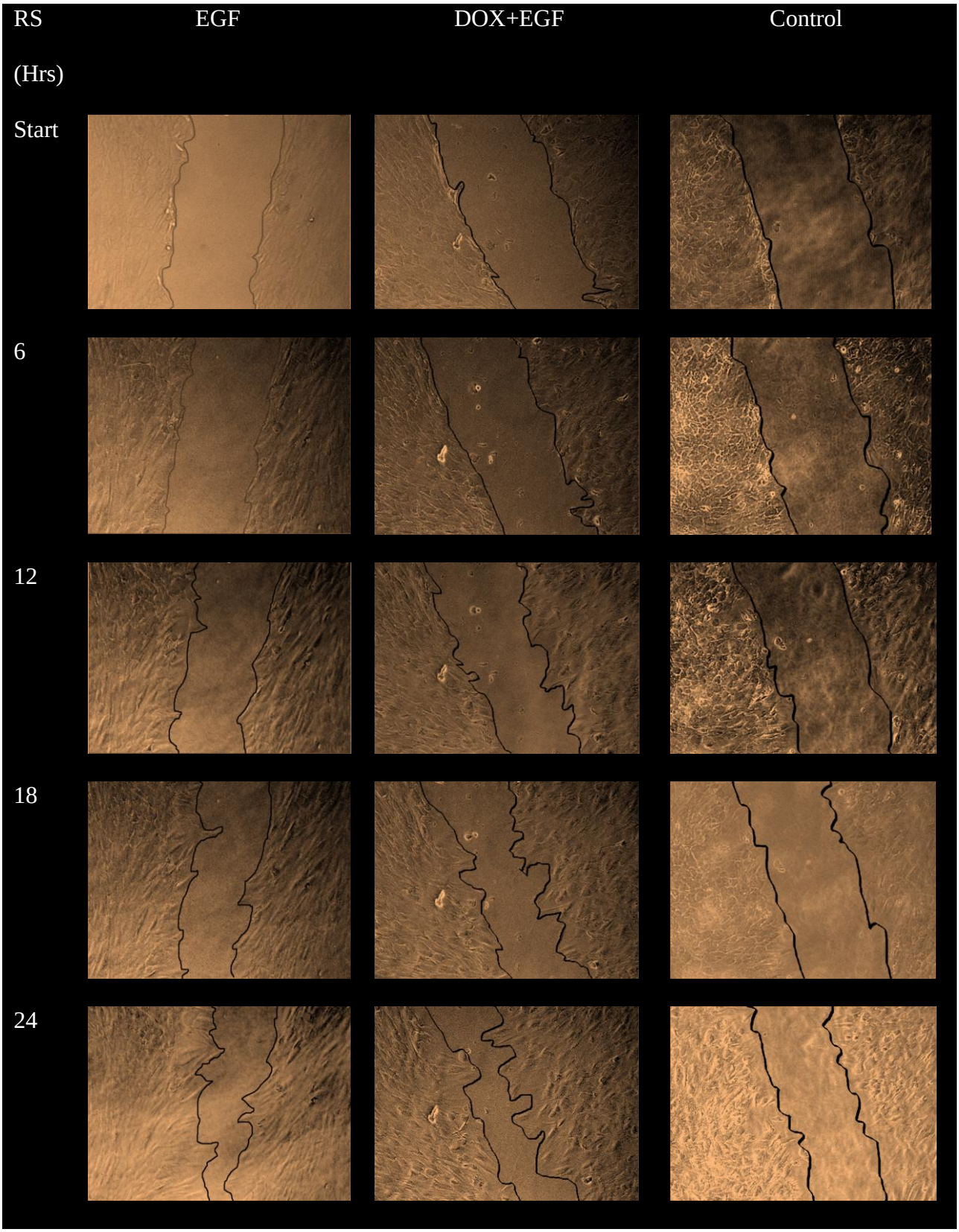
(iii) Control (pristine PU film).

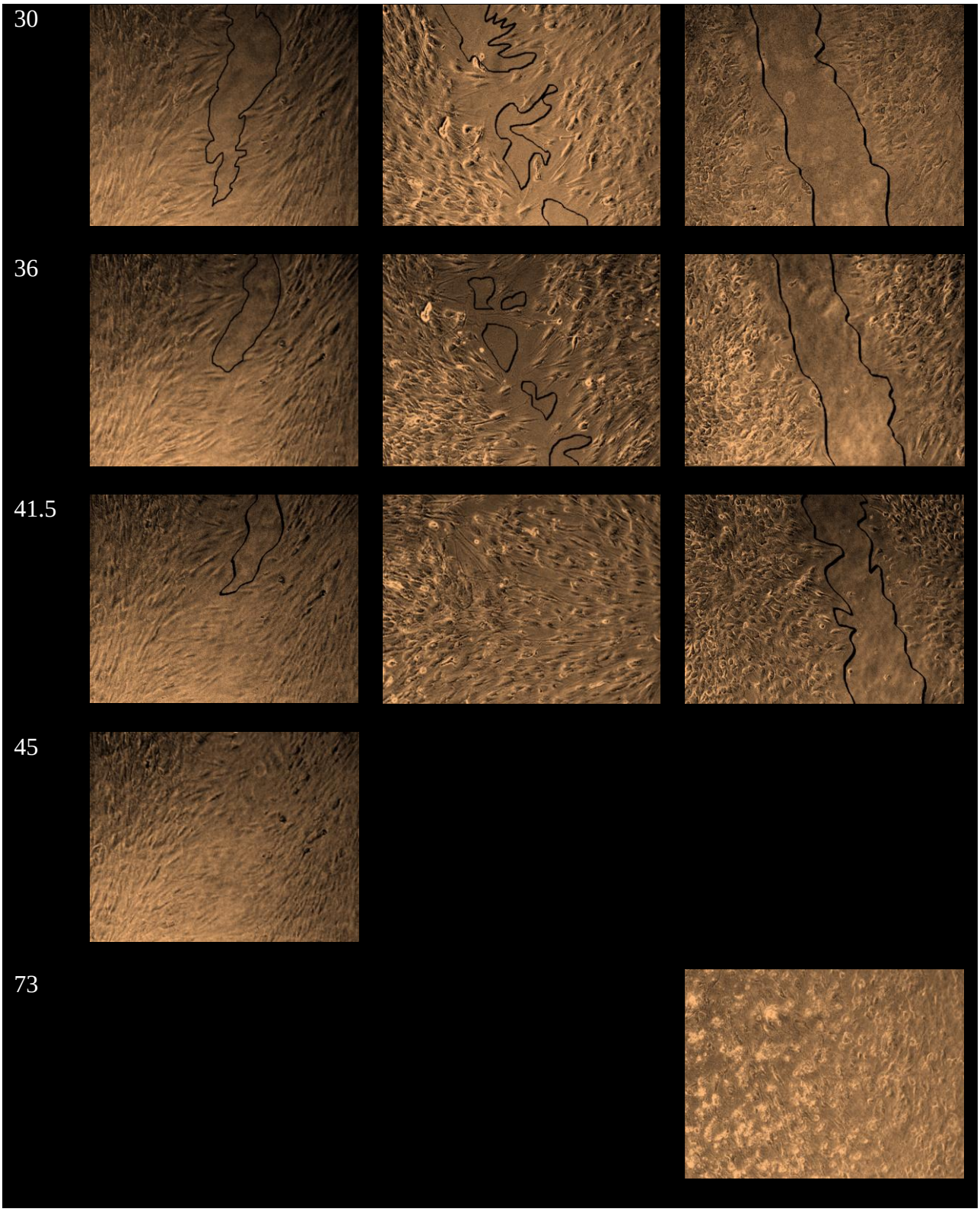
The films were laid above the cells containing media. Wound closure was measured under the microscope over a time period until the wound was completely closed. The migration of cells in all three cases is shown in **Figure 17.** The initial distance of the incision is almost the same in all cases. In case of the second treatment (EGF only), the migration rate up to 24 hours was very slow **(Figure 17b)** and after 24 hours the cells migrated rapidly at an rate of 45µm for every 6 hours to cover the incision in just 45 hours.

In previous part 01 of only EGF with proteases, the wound closed in just 27 hours because the absence of proteases created a clean environment for the EGF to induce

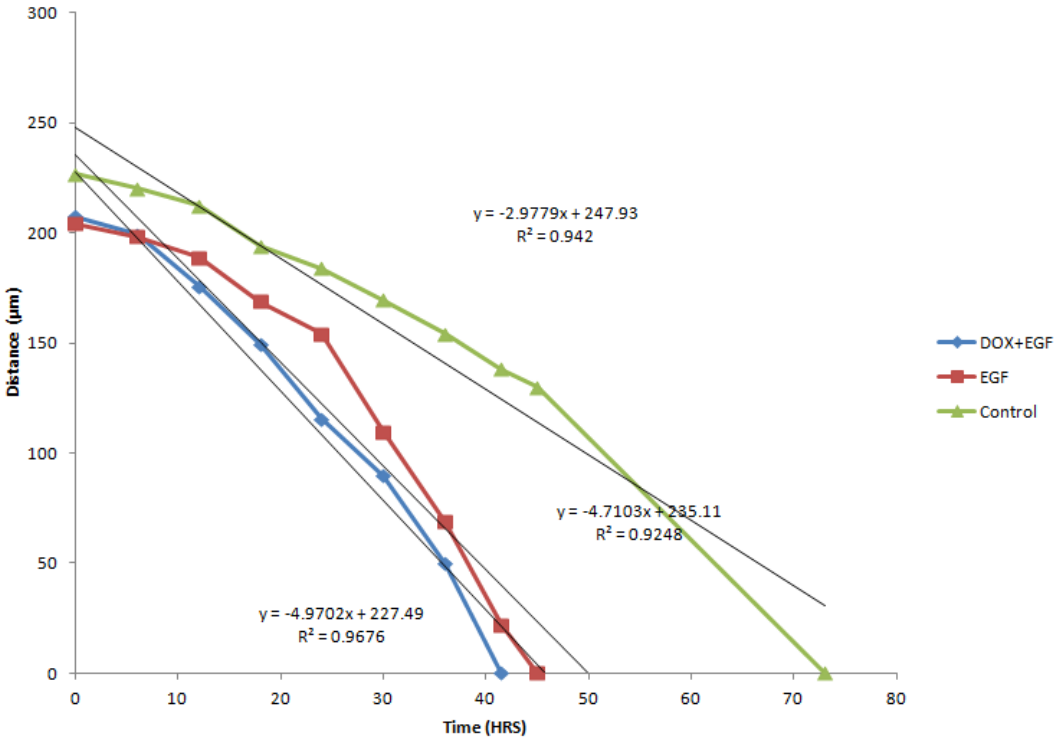
mitogenic effect on cells. However, in this experiment, in the presence of proteases, the degradation of released EGF by the proteases was observed in first 24 hours. After 24 hours, as the concentration of proteases is decreased and also due to the shelf life of MMP-9 began to decline, leaving behind a safe environment for the further released EGF to fasten the cell migration and proliferation. This effect can be seen in **Figure 17b** in the form of two slopes. In the first treatment, the released DOX first inhibited the available proteases, hence protected the protected the EGF from the exposure to proteases. This allowed the highly bioactive EGF induced mitogenic activity in cells and increased the cell migration rate. In the DOX+EGF treatment, the wound closed in just 41.5 hours, significantly faster than the EGF only treatment. Secondly, only around 5.5 ng of cumulative EGF was released in DOX+EGF treatment. Comparatively, in the EGF only treatment, significant change in the migration rate occurred when 23 ng of EGF was released and it took 45 hours in presence of proteases for the wound to close. Hence, the DOX+EGF treatment showed that a low amount of EGF was sufficient to accelerate cell migration faster than larger amounts of EGF. The movement in the control sample was very slow due to the absence of EGF.

Since a simple wound model was used in this experiment, one must be aware of its shortcomings in mimicking the real wound phenomenon. In chronic wounds, the proteases are generated regularly. In this simple wound model, proteases were added only once just before the start of the experiment. These proteases may degrade over time and with no further administration of proteases, the effects of the treatments in an environment where proteases would continue to generate could not observed.


RS
(Hrs)
EGF
DOX+EGF
Control
Start
6
12
18
24



(a)



(b)

Figure 17(a). Rate of Healing of a scratched wound model covered with DOX + EGF deposited PU film, only EGF deposited PU film and control - pristine PU film. **(b)** Wound gap as a function of healing duration, indicating fibroblast cell migration rate.

CHAPTER 5

Conclusion and Future Studies

Epidermal growth factor, a small protein crucial to wound healing, has been encapsulated in various biomedical materials, such as nanofibers and microspheres for sustained delivery. However, these technologies involve using organic solvent, or high electric voltage, or high mechanical stress, all of which may endanger the activity of EGF. In contrast, layer-by-layer assembly is a mild and effective technique to fabricate nano-scale multilayer's on surfaces. Large proteins such as DNA, BSA and FGF have been successfully encapsulated using LbL technique; however, there has been no report on the encapsulation of EGF using this technique. The challenge of this research is to encapsulate a small protein such as EGF using LbL assembly with its biological activity maintained during and after encapsulation.

To enhance the healing of wounds, continuous exposure of EGF to cells is necessary in order to achieve mitogenic activity of cells. Through encapsulation, a controlled release of bioactive EGF can maintain a continuous exposure of EGF to cells. Previous studies indicate that micrograms of released EGF enhanced the healing rate. Buckley et al. showed that a release of 4 ng/hour EGF accelerated wound healing. However, we speculate that as long as EGF retained its bioactivity through the encapsulation and release processes, even very limited amount of released bioactive EGF could significantly promote wound healing in the wound model. Our research results confirm that small amounts of highly bioactive EGF can stimulate cell migration and proliferation, expediting the wound closure and healing.

When EGF is released in a controlled manner, it provides continuous exposure of EGF to cells. Highly bioactive EGF induces mitogenic activity thereby expediting the wound closure. The first objective is to design a strategy to encapsulate EGF and release

EGF with retained bioactivity to enhance healing. We found that EGF could be encapsulated using LbL assembly on a functionalized surface having CH molecules. The assembly later released the encapsulated EGF from the film through the swelling of the film, indicating that the release can be tuned for further days depending upon the number of layers deposited onto the surface. We could release 23 ng of EGF in a controlled manner over seven days. We also quantified the bioactivity of EGF at 94% bioactive after 4 days of release. We interpret the data from our actin staining experiments as supporting evidence that released EGF has a biological activity that includes enhancement of the contractile machinery for cell locomotion. It should be noted that many types of F-actin subtypes are involved in the components of cell migration: formation of focal adhesions, extrusion of 'lamellopodia' at the leading edge of cells, and trailing edge retractions. The differences in F-actin distribution in our study are therefore consistent with both the increased migratory activity we observed, and the aggregation of cells in conditions which did not promote cell migration. Thus a strategy has been developed to design a biological wound dressing with a tunable release with the compromise of bioactivity. Maintaining the active state of the biological molecules and releasing these molecules at a rate determined by the state and depth of the wound facilitates closure and healing of chronic wounds.

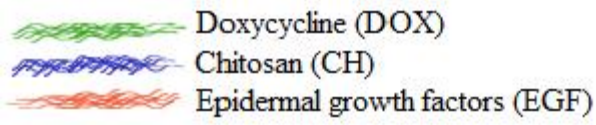
EGF can be released in a time dependent manner increasing the mitogenic activity of cells due to the continuous exposure; however, chronic wounds exhibit higher levels of MMP-9 protease that easily degrade released EGF making the wound environment deficient in EGF, preventing wound closure.

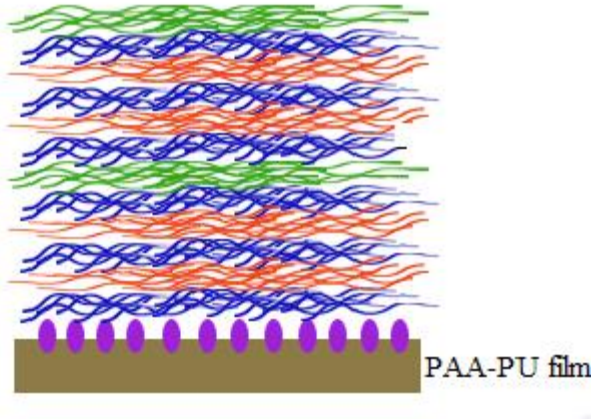
. Because MMP-9 is regenerated in chronic wounds, large amounts of EGF are required, which is not economical. The second major objective is to achieve both the functions of inhibiting the MMP-9 protease and using a lower amount of bioactive EGF, the protease inhibitor DOX is used to inhibit the protease and to protect the released EGF. In a clinical situation, proteases are very high in number and easily degrade the released EGF. So, it is a good idea to provide a safe environment for the later released EGF by first inhibiting the MMP-9 proteases. We used DOX as the protease inhibitor. Azocoll assay showed that the protease MMP-9 was inhibited by the DOX which was released; a safe environment was obtained for further released EGF; and DOX did not affect the bioactivity of the EGF encapsulated. Our wound model also suggested a cast amount of EGF was degraded when released in the presence of the MMP-9 protease. In contrast, when the released DOX inhibited the proteases, a lower amount of EGF still enhanced and speeded the wound closure.

These findings underline the importance of a controlled release of DOX to inhibit MMP-9 proteases along with a simultaneous release EGF to expedite wound healing. The findings open the doors for the development of novel strategies in wound healing.

Future studies:

(1) The DOX was deposited on top of EGF in the case of simple wound model. But in chronic wounds, MMP-9 proteases are regenerated, which pose a threat to the later released EGF. So in order to inhibit the regenerated MMP-9 DOX can be encapsulated at various layers as shown in the below scheme.





(2) EGF and DOX can also be encapsulated on surface modified nano-fibers, which provide more surface area and 3D embroidered in order to lower the distance between the wound and dressings.

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