Heparin functionalized core-shell type nanofabrics as a potent growth factor sequestering biomaterial for skin wound healing

アクシャット, ジョシー

Heparin functionalized core-shell type nanofabrics as a potent growth factor sequestering biomaterial for skin wound healing

December 2020

AKSHAT JOSHI

Table of Contents

List of abbreviations

GF: Growth Factor

PCL: Polycaprolactone

PLGA: poly (lactic-co-glycolic acid)

PG: PCL/gelatin co-spun nanofibrous membranes

VEGF: Vascular endothelial growth factor

PDGF: Platelet derived growth factor

bFGF: Basic fibroblast growth factor

Hep-PG: Heparin functionalized PG nanofibrous dressings

WVTR: Water vapor transmission rate

EGF: Epidermal growth factor

KGF: Keratinocyte growth factor

TGF-β: Transforming growth factor beta

HGF: Hepatocyte growth factor

KGM: Konjac glucomannan

DDS: Drug delivery systems

EDC: 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide

NHS: *N*-Hydroxysuccinimide

ECM: Extracellular matrix

GAGs: Glycosaminoglycans

PLLA: poly (l-lactic acid)

SEM: Scanning electron microscopy

TEM: Transmission electron microscopy

FTIR: Fourier transform infrared spectroscopy

Abstract

GFs are signaling molecules that are principle mediators in tissue regeneration. Biomaterial scaffolds employed as wound dressings are often hampered by their limitations to deliver GFs exogenously due to their instability and low half-life. Overdosing is often required, which raises further concerns as severe as carcinogenesis. Effective restoration of blood supply at the degenerative site while minimising the use of exogenous GFs remains a pressing challenge for engineered regenerative medicine.

The key to overcoming this challenge possibly lies in the better organisation and use of endogenous GFs, with an aim to abandon the use of exogenous proteins that are usually administrated at a supraphysiological level. The concentrations of endogenous pro-regenerative GFs can be sufficiently high to initiate angiogenesis at the regenerative site. A strategy to maximise the production and action of endogenous pro-angiogenic GFs at the wound site may become a new strategy of therapeutic angiogenesis without delivering exogenous proteins.

Considering such challenges, current research utilizes the exogenous and endogenous GFs sequestering ability of heparin functionalized PG nanofibrous dressings to facilitate synergistically driven tissue regeneration by utilizing combined therapeutic effect of exogenous and endogenous GFs, and thereby minimizing the sole dependency on exogenous GFs for tissue regeneration, that have severe concerns in terms of safety and efficacy. Since heparin is well-known for its ability to sequester numerous GFs, these fabricated heparin functionalized dressings showed ability to sequester and stabilize pro-regenerative GFs released at injury site and worked synergistically with exogenous loaded GFs to promote accelerated tissue regeneration.

Chapter 1

Overview of Dissertation

1.1 Motivation and objective

Since the discovery of insulin in the 1920s, protein-based therapeutics have been investigated for suitable routes of delivery and extended biological half-life[1]. Recently advanced techniques have been developed to enhance the efficiency of protein or peptide drugs for use in preventing and treating human injury and several life-threatening diseases. Growth factors (GFs) are attractive candidates because of their unique roles in signalling cells and regenerating tissues. There are currently over 6,000 clinical trials regarding the use of growth factors.

When using GFs as candidate drugs, controlled and therapeutic delivery of these proteins is of pivotal importance for medicinal and economical reasons. Particularly in the case of multiple protein delivery, the order, location and duration of the release of each protein need to be carefully designed for the most therapeutic benefit.

Current multi-protein delivery strategies mainly rely on hydrolysable scaffolds and thin films of protein-containing polymers, which cannot be programmed to respond to biological signals. For example, existing approaches to achieve either sustained release or sequential release of proteins rely on polymer hydrolysis or passive leaching. The Langer Laboratory at MIT pioneered the most successful example of this type of delivery, with proteins entrapped within PLGA scaffolds that are released as the polymer hydrolyses[2]. The Mooney group at Harvard University expanded this approach to allow sequential delivery of two proteins through differential entrapment methods[3]. Recent development includes the hybrid polymer matrices with different degrading rates, barrier

polymer films[4] for sequential delivery or orthogonal binding pairs to allow for multiple protein release, resulting in poor temporal control.

However, these developments for ordered delivery of multiple proteins have certain limitations. First, the release is driven by hydrolysis; therefore, it is not responsive to the biological milieu and may not be adaptive to the pathological development of tissues[5-7]. Second, in existing approaches, the incorporation of proteins into polymer matrices generally involves intense mixing and/or use of organic solvents; and only certain polymers are used. Such harsh chemical processes can easily denature the proteins, precluding their use as a general delivery vehicle[8-10]. Last, in previous attempts to achieve enzyme-responsive delivery of proteins from one scaffold, existing approaches are generally limited by the types and the number of available orthogonal-binding functional groups in the composition of the scaffold. Also, exogenous delivery increases the cost of treatment [11, 12] and has concerns over safety and efficacy[13-15].

In light of these developments and limitations, the current research aims to develop a biomaterial that have ability to sequester growth factors that are released at regenerative site and release them during the healing process to accelerate tissue regeneration with minimum dependency on exogenous delivery. In response to injury or ischaemia, the level of VEGF sharply increases[16, 17], and the platelets release ample PDGF in the early stages of wound healing[18]. Also, when a biomaterial is implanted, it also activates the local tissues and its elasticity and components trigger the surrounding cells to secrete many cytokines[19]. Importantly, macrophages, the principal mediator of inflammatory responses to injury or foreign bodies, are activated by injury signals, change their phenotypes and produce a cocktail of GFs at varying levels throughout this process[20-

22]. These cells are the primary source of many GFs during tissue regeneration. A strategy to maximise the production and action of endogenous pro-regenerative GFs at the wound site may become a new strategy of therapeutic angiogenesis without delivering exogenous proteins.

1.2 Research objective

The main target of this research is to develop nanotechnology based wound dressing that can have the ability to sequester endogenous and exogenous GFs with high efficiency and can utilize the synergistic effect of these exogenous and endogenous proteins to promote accelerated tissue regeneration. The primary objectives are listed below:

- To fabricate PG nanofibrous membranes that balances between mechanical characteristics and biocompatibility
- Characterization of PG co-spun nanofibrous membranes as wound dressings
- Heparinization of PG nanofibrous membranes (Hep-PG) and evaluation of their application as Novel Drug Delivery System (NDDS) to sequester endogenous and exogenous GFs
- Application as wound dressings to treat full-thickness wounds to obtain complete tissue regeneration with minimum scarring based on the therapeutic effect of these sequestered exogenous and endogenous GFs

1.3 Novelty of current study

Restoration of blood supply at regenerative site is often required at earlier stages of healing to attain proper regeneration at later stages. As for a wounded tissue, this restoration is accelerated through the delivery of exogenous growth factors that stimulate

the process of angiogenesis at initial stage that can lead to an effective tissue regeneration. The supply of GFs from exogenous sources is indispensable, as the endogenous ones often undergo early degradation in the protease rich environment at regenerative site.

Previous researches have basically relied on delivery of pro-angiogenic GFs individually or in combinations to enhance the process of angiogenesis. Also, many of current researches have proved that these pro-angiogenic GFs namely, VEGF, FGF and PDGF when used in combination can synergistically accelerate the process of angiogenesis at initial stages that have led to an effective and accelerated tissue regeneration [23-26]. So overall, considering the findings from previous studies, this current dissertation relies on delivering pro-angiogenic GFs by an ECM mimicking nanofibrous wound dressings. We believed that if these GFs combinations can be delivered, they can have strong synergistic effects to accelerate the process of angiogenesis, and thereby a quick and efficient regeneration can be achieved.

To achieve our target, we applied a novel biomaterial-based approach to sequester pro-angiogenic GFs into our nanofibrous dressing both from exogenous and endogenous source. Our fabricated dressing can retain these pro-angiogenic GFs at initial stages of healing and can deliver them at later stages that can lead to an accelerated tissue regeneration. We supplied FGF from exogenous source and VEGF and PDGF were sequestered by our matrix from endogenous sources. Overall, initially all three GFs were sequestered by our matrix and as healing progressed, delivery of these pro-angiogenic factors resulted in effective tissue regeneration. The schematic for such a phenomenon is shown below in figure 1-1.

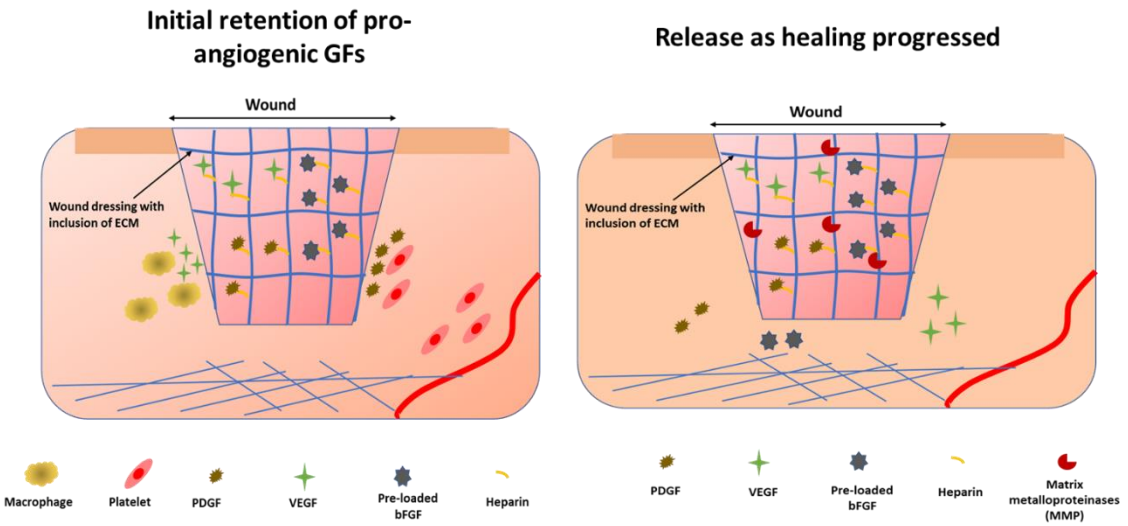


Figure 1-1: Retention and release mechanism by our dressing. FGF was supplied from exogenous source whereas PDGF and VEGF were sequestered from endogenous source. These sequestered GFs can be released from our dressings at later stages of healing that can synergistically promote efficient angiogenesis at initial stages and proper tissue regeneration at later stages.

We believe such accelerated tissue regeneration was possibly due to the synergistic effect of these pro-angiogenic GFs delivered by our dressings, that lead to quicker angiogenesis at initial stages and effective tissue regeneration at later stages. This was the first study till date to supply pro-angiogenic GFs with minimum dependency on exogenous delivery and utilizing GFs from endogenous source, which would have otherwise undergone degradation. We believe such findings can be valuable in the field of wound care and management, that has faced serious concerns related to efficacy and safety of delivering exogenous proteins.

1.4 Thesis outline

This thesis consists of 6 chapters. The chapters are arranged in order to achieve the basic objective of this study. The following paragraphs will give a glimpse of the work content of each chapter:

Chapter 1: of the thesis discusses about the background and objective of this research. It also highlights the novelty of this study and how this study was advantageous over current available techniques for fabrication of wound dressings

Chapter 2: of this thesis provides a comprehensive review on delivery systems for wound healing. It ushers in an introduction of wound healing and the mechanisms involved there forth outlining the physiology, mechanism of actions, wound types and wound management.

Chapter 3: in this chapter, co-electrospinning technique was optimized to fabricate core-shell type nanofabrics. By controlling various co-electrospinning parameters, uniform and bead-free PG nanofabrics were produced that showed excellent balance between mechanical properties and biocompatibility. Also, the fabricated nanofabrics met all the criterion for its application as wound dressings. Results showed that PG nanofabrics were an ideal material for application as wound dressings owing to their excellent mechanical properties that mimic the skin elasticity, enhanced biocompatibility due to presence of gelatin as shell component as well as ideal WVTR and surface wettability.

Chapter 4: in this chapter, the fabricated core-shell type nanofabrics were endowed with the ability for sequestering GFs, both exogenous and endogenous ones. For this, we conjugated our fabricated nanofabrics to heparin via EDC/NHS mediated reaction. These heparin functionalized core-shell type nanofabrics, called as Hep-PG, showed excellent

capability in sequestering GFs both exogenous and endogenous ones. Results showed tremendous application of heparin in sequestering GFs both exogenous and endogenous ones with high efficiency.

Chapter 5: in this chapter, the fabricated Hep-PG nanofabrics were employed as wound dressings to evaluate their potential in effective skin tissue regeneration. Endowed with properties of ideal wound dressings and capability in sequestering exogenous and endogenous GFs, Hep-PG nanofabrics promoted scarless and effective tissue regeneration within 14 days of treatment, by utilizing the synergistic effect between exogenously loaded bFGF and sequestered endogenous pro-regenerative GFs. Here we demonstrated a novel biomaterial-based approach with inclusion of heparin, to sequester GFs from exogenous and endogenous source, and how a synergistic effect of exogenous and endogenous GFs can lead to proper tissue regeneration. This was the first study till date which evaluated a combined role of exogenous and endogenous GFs based on heparin in achieving tissue regeneration.

Chapter 6: of this thesis gives a general conclusion and future study that can be incorporated to further advance the current study

1.5 List of Publications

Journal Papers

- 1. *Akshat Joshi*, Zhe Xu, Yasuhiro Ikegami, Kozue Yoshida, Yusuke Sakai, Akshay Joshi, Tejinder Kaur, Yosuke Nakao, Yo-ichi Yamashita, Hideo Baba, Shinichi Aishima, Neetu Singh, Hiroyuki Ijima, "Exploiting synergistic effect of externally loaded bFGF and endogenous growth factors for accelerated wound healing using heparin functionalized PCL/gelatin co-spun nanofibrous patches", *Chemical Engineering Journal*, https://doi.org/10.1016/j.cej.2020.126518, 404, 1-13, Article 126518, Accepted (2020/7/30). *Impact Factor: 11.35.*

- *Akshat Joshi*, Zhe Xu, Yasuhiro Ikegami, Soichiro Yamane, Masanori Tsurashima, Hiroyuki Ijima, Co-culture of mesenchymal stem cells and human umbilical vein endothelial cells on heparinized polycaprolactone/gelatin co-spun nanofibers for improved endothelium remodeling, International Journal of Biological Macromolecules, https://doi.org/10.1016/j.ijbiomac.2020.02.163, 151, 186-196, Accepted (2020/2/15), 2020.02. *Impact Factor: 5.5.*

- Jannatul Fardous, Yuji Omoso, *Akshat Joshi*, Kozue Yoshida, Md Kawchar Ahmad Patwary, Hiroyuki Ijima, "Development and characterization of gel-in-water nano-emulsion as a novel drug delivery system", Material Science and Engineering: C [Under review]. *Impact Factor: 6.1.*

Conference Papers:

- Sindhuja D E, *Akshat Joshi*, Akshay Joshi, Subha Narayan Rath, 'Two-cell bioprinting for bone tissue engineering application', TERMIS-EU 2017.06
- *Akshat, Joshi*, Zhu Xu, Nana Shirakigawa, Hiroyuki Ijima, 'Development of heparin immobilized PCL/gelatin core-shell type nanofibrous scaffolds: A potential candidate for Tissue Engineering Applications', 第 54 回九大生体材料・力学研究会, 2018.11
- Hiroyuki Ijima, *Akshat Joshi*, Zhe Xu, Nana Shirakigawa, 'Development of Functional Core-Shell Type Nanofibrous Scaffolds', JAACT 2018 Tsukuba, 2018.11
- *Joshi Akshat*, Zhe Xu, Shirakigawa Nana, Ijima Hiroyuki, 'Development of biomimetic PCL/gelatin core-shell type nanofibrous scaffolds immobilized with Heparin Sulfate for Tissue Engineering applications', 化学工学会 第 50 回秋季大会, 2018.09
- Xu Zhe, *Joshi Akshat*, Yasuhiro Ikegami, Nana Shirakigawa, Hiroyuki Ijima, Fabrication of artificial blood vessel using gelatin/PCL core-shell nanofiber, 第 55 回化学関連支部合同九州大会, 2018.06
- *Akshat Joshi*, Zhe Xu, Yasuhiro Ikegami, Dipul Chawla, Akshay Joshi, Tejinder Kaur, Hiroyuki Ijima. 'bFGF loaded Heparinized PCL/gelatin co-spun nanofabrics as growth factor delivery vehicle for accelerated wound healing', TERMIS EU-Chapter/Manchester 2020.05.

References

[1] T. Miao, J. Wang, Y. Zeng, G. Liu, X. Chen, Polysaccharide-based controlled release systems for therapeutics delivery and tissue engineering: from bench to bedside, Adv. Sci. 5(4) (2018) 1700513.

[2] O.S. Fenton, K.N. Olafson, P.S. Pillai, M.J. Mitchell, R. Langer, Advances in biomaterials for drug delivery, Adv. Mater. 30(29) (2018) 1705328.

[3] K. Lee, E.A. Silva, D.J. Mooney, Growth factor delivery-based tissue engineering: general approaches and a review of recent developments, J. Royal Soc. Interface 8(55) (2011) 153-170.

[4] J. Li, D.J. Mooney, Designing hydrogels for controlled drug delivery, Nat. Rev. Mater. 1(12) (2016) 1-17.

[5] B.B. Hsu, K.S. Jamieson, S.R. Hagerman, E. Holler, J.Y. Ljubimova, P.T. Hammond, Ordered and kinetically discrete sequential protein release from biodegradable thin films, Angew. Chem. Int. Ed. 53(31) (2014) 8093-8098.

[6] Y.-T. Lai, N.P. King, T.O. Yeates, Principles for designing ordered protein assemblies, Trends Cell Biol. 22(12) (2012) 653-661.

[7] M. Hu, L. Qian, R.P. Briñas, E.S. Lymar, J.F. Hainfeld, Assembly of nanoparticle–protein binding complexes: from monomers to ordered arrays, Angew. Chem. Int. Ed. 46(27) (2007) 5111-5114.

[8] Y. Luo, A. Lode, F. Sonntag, B. Nies, M. Gelinsky, Well-ordered biphasic calcium phosphate–alginate scaffolds fabricated by multi-channel 3D plotting under mild conditions, J. Mater. Chem. B 1(33) (2013) 4088-4098.

[9] J.L. Frandsen, H. Ghandehari, Recombinant protein-based polymers for advanced drug delivery, Chem. Soc. Rev. 41(7) (2012) 2696-2706.

[10] M.R. Battig, B. Soontornworajit, Y. Wang, Programmable release of multiple protein drugs from aptamer-functionalized hydrogels via nucleic acid hybridization, J. Am. Chem. Soc. 134(30) (2012) 12410-12413.

[11] G.A. Ksander, Y. Ogawa, G. Chu, H. McMullin, J. Rosenblatt, J. McPherson, Exogenous transforming growth factor-beta 2 enhances connective tissue formation and wound strength in guinea pig dermal wounds healing by secondary intent, Ann. Surg. 211(3) (1990) 288.

[12] U. Park, M.S. Lee, J. Jeon, S. Lee, M.P. Hwang, Y. Wang, H.S. Yang, K. Kim, Coacervate-mediated exogenous growth factor delivery for scarless skin regeneration, Acta Biomater. 90 (2019) 179-191.

[13] L. Balazs, J. Okolicany, M. Ferrebee, B. Tolley, G. Tigyi, Topical application of the phospholipid growth factor lysophosphatidic acid promotes wound healing in vivo, Am. J. Physiol. Regul. Integr. Comp. Physiol. 280(2) (2001) R466-R472.

[14] N.R. Johnson, Y. Wang, Controlled delivery of heparin-binding EGF-like growth factor yields fast and comprehensive wound healing, J. Control. Release 166(2) (2013) 124-129.

[15] S. Barrientos, O. Stojadinovic, M.S. Golinko, H. Brem, M. Tomic-Canic, Growth factors and cytokines in wound healing, Wound Repair Regen. 16(5) (2008) 585-601.

[16] E. Ergenekon, K. Gücüyener, D. Erbaş, S. Aral, E. Koç, Y. Atalay, Cerebrospinal fluid and serum vascular endothelial growth factor and nitric oxide levels in newborns with hypoxic ischemic encephalopathy, Brain Dev. 26(5) (2004) 283-286.

[17] Y. Feng, Q. Li, D. Wu, Y. Niu, C. Yang, L. Dong, C. Wang, A macrophage-activating, injectable hydrogel to sequester endogenous growth factors for in situ angiogenesis, Biomaterials 134 (2017) 128-142.

[18] D.W. Long, N.R. Johnson, E.M. Jeffries, H. Hara, Y. Wang, Controlled delivery of platelet-derived proteins enhances porcine wound healing, J. Control. Release 253 (2017) 73-81.

[19] R. Sridharan, A.R. Cameron, D.J. Kelly, C.J. Kearney, F.J. O'Brien, Biomaterial based modulation of macrophage polarization: a review and suggested design principles, Mater. Today 18(6) (2015) 313-325.

[20] Y. Niu, Q. Li, Y. Ding, L. Dong, C. Wang, Engineered delivery strategies for enhanced control of growth factor activities in wound healing, Adv. Drug Deliver. Rev. 146 (2019) 190-208.

[21] J.V.D. Dolder, R. Mooren, A.P. Vloon, P.J. Stoelinga, J.A. Jansen, Platelet-rich plasma: quantification of growth factor levels and the effect on growth and differentiation of rat bone marrow cells, Tissue Eng. 12(11) (2006) 3067-3073.

[22] V.E. Santo, A.R.C. Duarte, E.G. Popa, M.E. Gomes, J.F. Mano, R.L. Reis, Enhancement of osteogenic differentiation of human adipose derived stem cells by the controlled release of platelet lysates from hybrid scaffolds produced by supercritical fluid foaming, J. Control. Release 162(1) (2012) 19-27.

[23] G. Sufen, Y. Xianghong, C. Yongxia, P. Qian, bFGF and PDGF-BB have a synergistic effect on the proliferation, migration and VEGF release of endothelial progenitor cells, Cell Biol. Int. 35(5) (2011) 545-551.

[24] R. Cao, E. Bråkenhielm, R. Pawliuk, D. Wariaro, M.J. Post, E. Wahlberg, P. Leboulch, Y. Cao, Angiogenic synergism, vascular stability and improvement of hind-limb ischemia by a combination of PDGF-BB and FGF-2, Nat. Med. 9(5) (2003) 604-613.

[25] Y. Bai, L. Bai, J. Zhou, H. Chen, L. Zhang, Sequential delivery of VEGF, FGF-2 and PDGF from the polymeric system enhance HUVECs angiogenesis in vitro and CAM angiogenesis, Cell Immunol. 323 (2018) 19-32.

[26] Y. Feng, Q. Li, D. Wu, Y. Niu, C. Yang, L. Dong, C. Wang, A macrophage-activating, injectable hydrogel to sequester endogenous growth factors for in situ angiogenesis, Biomaterials 134 (2017) 128-142.

Chapter 2: Wound healing and Growth Factors

2.1 Wound healing

Healing process refers to the substitution of destroyed or damaged tissues by newly created tissues by a living organism [1]. Healing is defined in a sequential process of physical attributes (phases) which constitute the process of post-trauma repair. The epidermis (surface layer) and dermis (deeper layer) in fully intact skin form a barrier against the external environment [2, 3]. This process is divided into predictable phases: blood clotting (hemostasis), inflammation, tissue growth (cell proliferation), and tissue remodeling (maturation and cell differentiation).

Not only the wound healing process is complex but also its fragile, and it is prone to disruptions or failure which results in the formation of chronic wounds. Factors contributing to non-healing chronic wounds are diabetes, venous or arterial disease, infection, and metabolic deficiencies of old age [3].

Wound treatment, by cleaning and protection from reinjuries or infection, facilitates and enhances wound healing process. It can range from the simplest first aid to entire nursing specialties, such as wound, ostomy, and continence nursing and burn centre care, depending on the needs of each patient.

2.2 Phases of wound healing

In an organized way, the stages of wound healing proceed and follow four processes: hemostasis, inflammation, proliferation and maturation. While the stages of wound healing are linear, depending on internal and external patient circumstances, wounds may progress backward or forward. The four wound healing stages are:

1. Hemostasis: Hemostasis is the process of the wound being closed by clotting. When blood leaks out of the body [4], hemostasis begins. The first step of hemostasis is when blood vessels constrict to restrict the blood flow. Next, platelets stay together in order to close the gap in the wall of the blood vessel. Coagulation eventually occurs and reinforces the platelet plug with fibrin threads that are like a molecular binding agent. The hemostasis phase of wound healing tends to occur very swiftly. The platelets adhere to the sub-endothelium surface within seconds of the rupture of a blood vessel's epithelial wall. After that, in about sixty seconds, the first fibrin strands begin adhering. As the fibrin mesh begins, the blood is transformed from liquid to gel through pro-coagulants and the release of prothrombin. In the wound area, the formation of a thrombus or clot keeps the platelets and blood cells trapped. In the stages of wound healing, the thrombus is usually important but becomes a problem if it detaches from the vessel wall and passes through the circulatory system, possibly causing a stroke, pulmonary embolism or heart attack.

2. Inflammatory Phase: Inflammation is the second stage of wound healing which starts right after the injury when the damaged blood vessels leak transudate (made of water, salt, proteins) causing localized swelling. Inflammation also regulates and prevents bleeding and infection at injury site [5]. The engorgement of the fluid allows the healing and repair cells to move to the wound site. During the inflammatory process, weakened cells, viruses, and bacteria are removed from the wound area. The swelling, heat, pain and redness commonly seen during this stage of wound healing are produced by these white blood cells, growth factors, nutrients and enzymes. Inflammation is a natural part of the wound healing process and only problematic if prolonged or excessive.

3. Proliferative phase: The proliferative process of healing process is when the wounds are rebuilt with new tissue which is composed of collagen and extracellular matrix. In the proliferative process, the wound contracts as new tissues are built [6]. In addition, a new network of blood vessels must be built so that the granulation tissue can be stable and receive adequate oxygen and nutrients. By grasping the wound edges and drawing them together using a mechanism similar to that of smooth muscle cells, myofibroblasts cause the injury to contract. Granulation tissue is pink or red and irregular in texture in healthy stages of wound healing. Moreover, healthy granulation tissue does not bleed easily. A symptom of infection, ischemia, or weak perfusion may be dark granulation tissue. Epithelial cells resurface the injury in the final step of the proliferative stage of wound healing. It is important to note that epithelialization occurs faster when wounds are kept moist and hydrated. Generally, they can retain proper tissue humidity to maximize epithelialization when occlusive or semi occlusive dressings are applied within 48 hours of injury.

4. Remodeling phase: Often called the maturation stage of wound healing, the maturation process is when collagen is remodeled from type III to type I and the wound completely closes [7]. Apoptosis, or programmed cell death, eliminates cells that have been used to heal the wound, but are no longer required. It is disorganized as collagen is laid down during the proliferative process, and the wound is dense. Collagen is positioned along tension lines during the maturation process and water is reabsorbed to enable the collagen fibers to lie closer together and cross-link. Cross-linking of collagen decreases scar thickness and makes the surface region of the wound stronger. Remodeling usually starts around 21 days after an accident and can continue for a year or more. Even with cross-

linking, healed wound areas appear to be weaker than uninjured tissue, typically only possessing 80 percent of the tensile strength of unwounded tissue.

The stages of wound healing are a complex and fragile process. In the stages of wound healing, failure to progress may lead to chronic wounds. Venous disease, infection, diabetes and elderly metabolic deficiencies are factors which lead to chronic wounds. By keeping wounds moist, clean and safe from reinjuries and infection, diligent wound care will speed up the stages of wound healing.

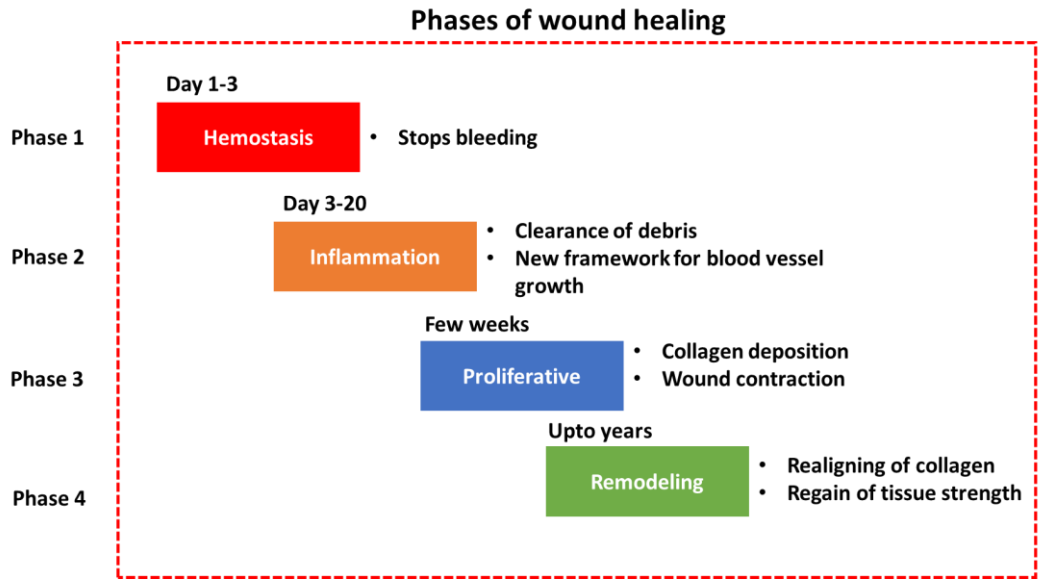


Figure 2-1: Phases of wound healing demonstrated pictorially

2.3 Growth factors in wound healing

Wound healing is a complex biological process in which a number of cells, including fibroblasts, myofibroblasts, smooth muscle cells, endothelial cells, keratinocytes and immune cells, involve cellular interactions. Numerous factors, such as growth factors, hormones, blood components and second messengers, mediate these interactions.

For wound healing, several growth factors which are released at the wound site are presumed to be required. The GFs mainly involved in skin wound healing are broadly classified into two categories:

2.3.1 Pro-angiogenic or pro-regenerative growth factors

For the healing of skin wounds and the regeneration of certain types of tissue, such as bone and skeletal muscle, the development of functional blood vessels at the injury site is essential [8-10]. The newly developed vasculature provides oxygen and nutrients to sustain cell growth and metabolism. Basically, pro-angiogenic growth factors are growth factors that are involved in supporting the development of vasculature at the regenerative site. These growth factors are also known as pro-regenerative factors, since vasculature is the most essential aspect for initiating the healing process. They are categorized as shown in table below:

Table 2-1: Pro-regenerative GFs and their role in wound healing process. It can be clearly seen from table 2-1 that pro-regenerative GFs are utilized in almost all phases of healing.

GF	Cells	Function	References
VEGF	Platelets Neutrophils Macrophages Endothelial cells Fibroblasts	• Granulation tissue formation • Stimulation of vasculogenesis and angiogenesis	[11-22]
FGF	Keratinocytes Mast cells Fibroblasts Endothelial cells Smooth muscle cells	• Granulation tissue formation • Re-epithelization • Matrix formation and remodelling • Angiogensis	[23-26]
PDGF	Platelets Neutrophils Macrophages Endothelial cells Fibroblasts	• Granulation tissue formation • Re-epithelization • Matrix formation and remodeling	[27-29]

2.3.2 Other growth factors

These include other growth factors that are involved in the healing process. Though their role is inferior as compared to pro-regenerative GFs, their utilization is indispensable for proper tissue regeneration. They are majorly classified as shown in table below:

Table 2-2: Other GFs and their role in wound healing process. Their role in healing process is supporting and less significant compared to pro-regenerative GFs [43].

GF	Cells	Function	References
TGF-β	Platelets Keratinocytes Macrophages Lymphocytes Fibroblasts	• Inflammation • Fibroblast proliferation • ECM remodelling • Angiogenesis	[30-36]
EGF	Platelets Macrophages Keratinocytes	• Re-epithelization	[37-41]
KGF	Fibroblasts	• Re-epithelization	[42]

2.4 Sequestering GFs to biomaterials

Proteins secreted by cells that govern several biological processes are GFs. Although they have long been proposed as potent therapeutic agents, due to their short half-lives in biological environments, their administration in a soluble form has proven costly and ineffective. In regenerative medicine strategies, biomaterial-based methods are increasingly pursued as alternatives to boost the effectiveness or, preferably, substitute the need for exogenous administration of GFs. Overall, the numerous GF sequestering systems built so far have remarkably enhanced the operation of GFs at reduced doses and, in some cases, completely avoided the need for their exogenous administration to direct cell fates. Thus, these bioinspired principles allow the rational exploration in regenerative medicine of the full therapeutic potential of GFs.

2.4.1 Strategies for incorporating growth factors into biomaterials

Various biomaterial-based approaches to deliver GFs for tissue engineering and regenerative medicine are broadly classified as:

1. Conventional Controlled Release Systems: The goal of these strategies was to improve the efficiency of GF action on cells by allowing localized administration, reducing variability in GF levels over time, achieving cell activation signal persistence, and resolving diffusion constraints on soluble delivery [43, 44]. Conventional release strategies depend on the delivery of exogenously loaded GFs via a 3D matrix or porous scaffold. [45]. It is important to understand the physicochemical properties, such as molecular weight, isoelectric point, and site of action when designing a vehicle for GF delivery [46].

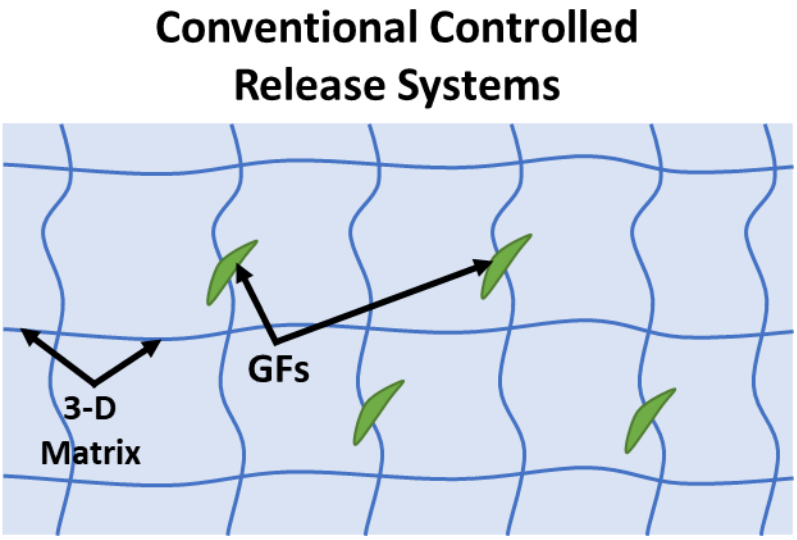


Figure 2-3: Pictorial representation of conventional release system. GFs are directly encapsulated into a 3-D polymeric matrix.

Challenges of such systems: Despite the continued developments in this form of controlled release systems, there are numerous aspects of previously mentioned GF

signaling mechanisms that are not taken into consideration. They constitute an advanced delivery mechanism for recombinant GF variants, enabling some problems of soluble bolus administration to be bypassed by a more sustained and prolonged release. The dependence on physical trapping and diffusion mechanisms, however, makes it difficult to change the conduct of release, particularly when considering several molecules in a single application. In addition, the multiple effects, such as sequestering and solid-phase presentation, resulting from the micro-environmental control of GF action are generally not present [47].

2. Material with covalently bound GFs: Covalent binding of GFs to a supporting biomaterial presents several advantages over previously discussed alternatives. First, over time, the proteins do not disperse into the surrounding medium. Combined with the enhancement of GF activity resulting from their solid-phase presentation, this enables the use of even lower doses to achieve similar biological effects. Second, they are less vulnerable to hydrolytic and enzymatic degradation by tethering proteins to a strong support, helping to prolong their function. Moreover, it is possible to achieve more accurate patterning and spatial organization of GFs in these systems. One of the approaches is to utilize NHS activated carboxylic groups of 3-d matrix that can react with the amine groups of proteins [48]. Other approaches utilize thiol chemistry, which is a biocompatible and relatively efficient way to link GFs to solid phase supports by taking advantage of existing cysteines in the protein. Several groups explored it to conjugate VEGF to scaffolding biomaterials designed to promote vasculogenesis, a key process to increase graft survival [49, 50]. Another highly biocompatible conjugation alternative is the use of click chemistry, particularly copper-free reactions. For example, strain-promoted azide–alkyne cycloaddition (SPAAC) has been used to couple azide-modified

EGF to primary amines in collagen, by means of dibenzo cyclooctyne (DBCO)–sulfo-N-hydroxysuccinimide (NHS) ester [51].

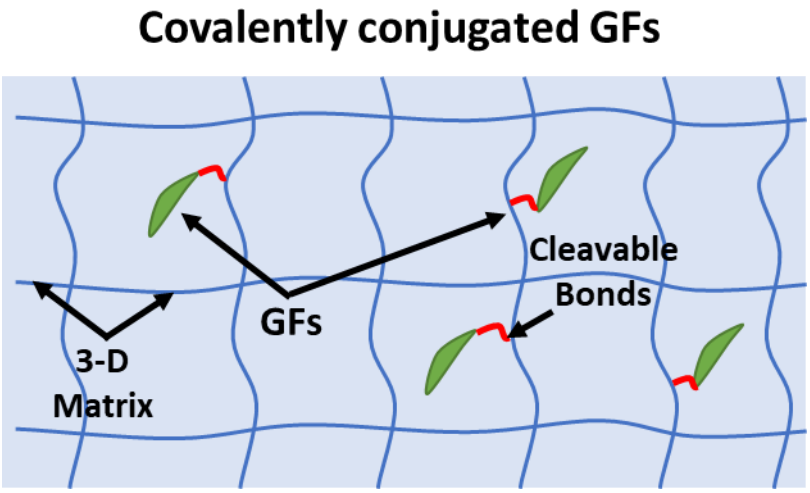


Figure 2-4: Pictorial representation of above scheme. GFs are included into 3-D matrix through cleavable covalent bonds. Such system ensures more control and spatial organization of GFs compared to conventional systems.

Challenges of this technique: They have the benefits of stabilizing and retaining GF on the scaffold supplied by covalent linking, while engineering their programmed release according to the stimuli provided. Thus, these systems can capture, to some degree, the complex actions of the natural ECM, and hold great promise for the near future. However, these advancements have been possible at the expense of a growing degree of complexity and related costs. In addition to the fact that they are still dependent on the inclusion of recombinant GFs and considering their previously stated disadvantages, it is important to consider whether large-scale development is feasible and economically viable. Indeed, their translation perspectives into commercial and clinical applications may still be far off, despite recent advances.

3. Platforms with Inclusion of ECM Components: Other research lines have been established to avoid the challenges associated with chemical reactions for covalent coupling and the increasingly complex biomaterials needed for controllable release. In order to effectively sequester these molecules from solution and present them at their surface for contact with cells, they have based on platforms with increased affinity for GFs. The way ECM and GFs interact in their natural environments, where noncovalent interactions predominate, is typically inspired by these biomimetic strategies.

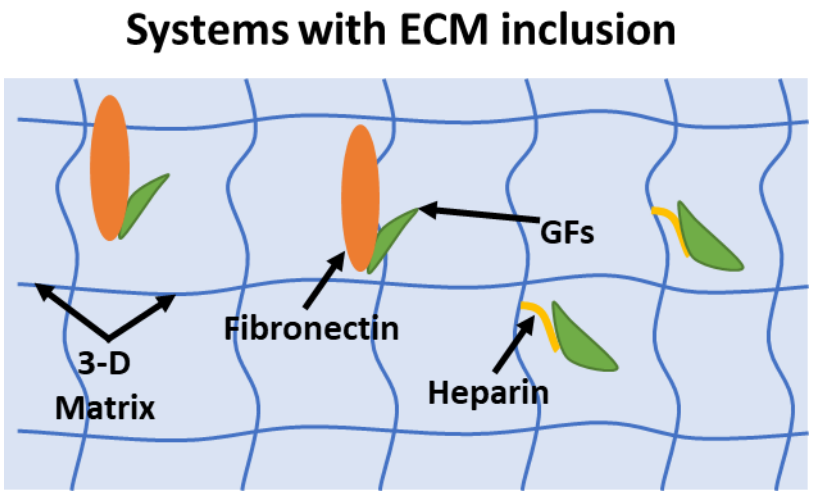


Figure 2-5: Pictorial representation of above scheme. GFs are directly sequestered by such systems through ECM components like fibronectin or heparin that are included into 3-D matrix. Such systems can recapitulate sequester and release mechanism of ECM.

Till date this technique is most successful method for delivering GFs as it recapitulates ECM mechanism. Moreover, studies have used this technique wherein, regeneration can be achieved with no dependency on exogenous delivery, since these systems can sequester locally produced endogenous GFs (sequester) and release them during the healing process, thus recapitulating ECM functions. The use of easily accessible natural

components such as heparin and fibronectin constitute a highly versatile approach to increase effectiveness and, therefore, reduce the required doses of administered GFs.

2.4.2 Various researches that have utilized above techniques for sequestering GFs to biomaterials for effective tissue regeneration

The table below shows few of the current researches that have utilized different biomaterial-based approach for GF sequestration and delivery employed for different tissue engineering applications.

Table 2-3: GF sequestration and delivery for biomedical application

Matrix	GFs	Immobilization approach	Biological effect	Application	GF Source	Ref
PLGA scaffold	VEGF	Physical encapsulation	VEGF released can maintain its activity for over 2 weeks	Bone regeneration	Exogenous	52
Alginate scaffold	VEGF, PDGF, TGF-β	Physical encapsulation	Multiple GF releasing alginate scaffolds promoted angiogenesis in-vivo	Angiogenesis	Exogenous	53
Gelatin hydrogel	bFGF	Physical encapsulation	Promotion of scarless acute vocal fold regeneration	Vocal fold regeneration	Exogenous	54
Diblock co-polymer matrix	HGF	Physical encapsulation	ECM synthesis and vocal fold regeneration	Vocal fold regeneration	Exogenous	55
PLGA scaffolds	VEGF	Covalent immobilization	Enhanced angiogenesis	Angiogenesis	Exogenous	56
Fibrin microthreads	HGF	Covalent immobilization	Enhanced myofibroblast proliferation	Skeletal muscle regeneration	Exogenous	57
Gelatin hydrogel	VEGF	Covalent immobilization	Neurite extension	Peripheral nerve regeneration	Exogenous	58
Chitosan-collagen hydrogel	TGF-β	Covalent immobilization	Scaffolds with controlled release of GFs promoted chondrogenesis	Cartilage regeneration	Exogenous	59
ECM proteins	VEGF, FGF, PDGF, TGF-β	ECM inclusion	Enhanced wound healing	Wound healing	Exogenous	60
EUP-3/ gelatin Hydrogel sponge	PDGF	ECM inclusion	Enhanced wound healing due to sequestered endogenous PDGF	Wound healing	Endogenous	61
KGM-heparin hydrogel	PDGF, VEGF, bFGF	ECM inclusion	Effective angiogenesis due to sequestered endogenous GFs	Wound healing	Endogenous	62

As seen from above table, different polymeric matrices were employed for GF delivery. Till date most researches rely on exogenous GFs delivery through different approaches. Only few current researches have employed endogenous GFs power for obtaining effective tissue regeneration. None of the research till date have exploited the combined role of exogenous and endogenous GFs, and how such systems can promote synergistically mediated tissue regeneration by utilizing combined effects of exogenous and endogenous GFs. Considering such literature, it was our objective to design a GF sequestering biomaterial that can utilize GFs from both exogenous and endogenous sources, and to exploit how a synergistic effect of GFs from exogenous and endogenous source can promote an effective tissue regeneration.

2.5 Conclusion

Considering the above-mentioned challenges and advancement, in our current research we are utilizing nanofiber-based DDS system for sequestering GFs both form external source and the locally produced endogenous ones and utilize their synergistic therapeutic effect to promote proper tissue regeneration.

To achieve such target, we considered three parameters that we believe can be considered prerequisite for fabrication of an ideal dressing. Firstly, to have minimum inflammatory response post transplantation and to facilitate proper cellular migration during the application, the dressing should closely mimic the structure of ECM. Skin is mostly composed of collagen, which have fibril like structure ranging up to few 100 of nanometres. To mimic such structure, nanofibrous dressings were fabricated. Further, to ensure closeness to skin ECM along with proper mechanical properties to allow ease of handling, co-axial electrospinning technique was adopted for dressing fabrication

wherein gelatin coated PCL nanofibrous patches with fiber diameter ranging few 100 of nanometers were fabricated. We chose PCL as core component due to its good mechanical properties whereas gelatin was chosen as shell component, to ensure the dressings closely mimic skin ECM, as gelatin is a derivative of collagen. Moreover, gelatin is cost effective and available in abundant compared to collagen.

Secondly, the dressings should have the capability to stabilize the endogenous GFs by sequestering them. If biomaterials employed as wound dressings have this property, the therapeutic effect of endogenous GFs can be utilized, and tissue regeneration can be achieved with minimum dependency on exogenous GFs delivery. To endow nanofabrics with endogenous GFs sequestering capability, surface functionalization with heparin was achieved via EDC/NHS coupling, due to its well-known ability in binding and enhancing the function of heparin binding GFs both in-vitro and in-vivo.

Thirdly, apart from being ECM mimicking and having capability to sequester endogenous GFs, the dressings should itself have therapeutic effect to enhance the tissue regeneration. To endow therapeutic capability into our fabricated dressings, we loaded limited amount of exogenous bFGF. bFGF was chosen as a candidate for exogenous delivery due to its well-known property to be involved in almost all phases of healing. Also, bFGF is known for its ability to promote scarless tissue regeneration when administered exogenously and has been clinically proven.

These fabricated nanofabrics were then employed as wound dressings to treat full-thickness wound using in-vivo rat model. By observing the morphology of wounds and through histological examinations, we showed that our nanofiber matrices not only accelerated the healing process, but also ensured scarless tissue regeneration. The

synergistic effect between externally loaded bFGF and sequestered endogenous GFs worked well, and we could obtain an accelerated tissue regeneration. To best of our knowledge, this is first report on the combined effect of endogenous and exogenous GFs in promoting proper tissue regeneration. We believe such valuable findings can find its application towards fabrication of ideal wound dressings.

References

[1] A.J. Singer, R.A. Clark, Cutaneous wound healing, N. Engl. J. Med. 341 (10) (1999) 738–746.

[2] S. Rieger, H. Zhao, P. Martin, K. Abe, T.S. Lisse, The role of nuclear hormone receptors in cutaneous wound repair, Cell Biochem Funct. 33(1) (2015) 1-13.

[3] G.C. Gurtner, S. Werner, Y. Barrandon, M.T. Longaker, Wound repair and regeneration, Nature 453 (7193) (2008) 314.

[4] H. Rasche, Haemostasis and thrombosis: an overview, European heart journal supplements 3 (suppl_Q) (2001) Q3-Q7.

[5] K.S. Midwood, L.V. Williams, J.E. Schwarzbauer, Tissue repair and the dynamics of the extracellular matrix, Int. J. Biochem. Cell Biol. 36(6) (2004) 1031-1037.

[6] N.N. Nissen, P. Polverini, A.E. Koch, M.V. Volin, R.L. Gamelli, L.A. DiPietro, Vascular endothelial growth factor mediates angiogenic activity during the proliferative phase of wound healing, Am. J. Pathol. 152(6) (1998) 1445.

[7] L.M. Morton, T.J. Phillips, Wound healing and treating wounds: Differential diagnosis and evaluation of chronic wounds, J. Am. Acad. Dermatol. 74(4) (2016) 589-605.

[8] T. Shantha Kumar, K. Soppimath, S. Nachaegari, Novel delivery technologies for protein and peptide therapeutics, Curr. Pharm. Biotechnol. 7(4) (2006) 261-276.

[9] R. Langer, J. Folkman, Polymers for the sustained release of proteins and other macromolecules, Nature 263(5580) (1976) 797-800.

[10] T.P. Richardson, M.C. Peters, A.B. Ennett, D.J. Mooney, Polymeric system for dual growth factor delivery, Nat. Biotechnol. 19(11) (2001) 1029-1034.

[11] N. Ferrara, Molecular and biological properties of vascular endothelial growth factor, J. Mol. Med. 77(7) (1999) 527-543.

[12] N. Ferrara, Vascular endothelial growth factor and the regulation of angiogenesis, Recent Prog. Horm. Res. 55 (2000) 15.

[13] N. Ferrara, Role of vascular endothelial growth factor in regulation of physiological angiogenesis, Am. J. Physiol 280(6) (2001) C1358-C1366.

[14] P.J. Taub, L. Silver, H. Weinberg, Plastic surgical perspectives on vascular endothelial growth factor as gene therapy for angiogenesis, Plast. Reconstr. Surg. 105(3) (2000) 1034-1042.

[15] P.S. Briquez, L.E. Clegg, M.M. Martino, F.M. Gabhann, J.A. Hubbell, Design principles for therapeutic angiogenic materials, Nat. Rev. Mater. 1 (1) (2016) 15006.

[16] M.N. Svendsen, K. Werther, H.J. Nielsen, P.E.G. Kristjansen, VEGF and tumour angiogenesis. Impact of surgery, wound healing, inflammation and blood transfusion, Scand. J. Gastroenterol. 37(4) (2002) 373-379.

[17] D.A. Redmer, V. Doraiswamy, B.J. Bortnem, K. Fisher, A. Jablonka-Shariff, A.T. Grazul-Bilska, L.P. Reynolds, Evidence for a role of capillary pericytes in vascular growth of the developing ovine corpus luteum, Biol. Reprod. 65(3) (2001) 879-889.

[18] J.M. Wood, Inhibition of vascular endothelial growth factor (VEGF) as a novel approach for cancer therapy, MEDICINA-BUENOS AIRES- 60 (2000) 41-47.

[19] M. Detmar, L.F. Brown, K.P. Claffey, K.-T. Yeo, O. Kocher, R.W. Jackman, B. Berse, H.F. Dvorak, Overexpression of vascular permeability factor/vascular endothelial growth factor and its receptors in psoriasis, J. Exp. Med. 180(3) (1994) 1141-1146.

[20] T.R. Howdieshell, D. Callaway, W.L. Webb, M.D. Gaines, C.D. Procter Jr, J.S. Pollock, T.L. Brock, P.L. McNeil, Antibody neutralization of vascular endothelial growth factor inhibits wound granulation tissue formation, J. Surg. Res. 96(2) (2001) 173-182.

[21] A.Y. Sheikh, J.J. Gibson, M.D. Rollins, H.W. Hopf, Z. Hussain, T.K. Hunt, Effect of hyperoxia on vascular endothelial growth factor levels in a wound model, Arch Surg. 135(11) (2000) 1293-1297.

[22] D.S. Steinbrech, M.T. Longaker, B.J. Mehrara, P.B. Saadeh, G.S. Chin, R.P. Gerrets, D.C. Chau, N.M. Rowe, G.K. Gittes, Fibroblast response to hypoxia: the relationship between angiogenesis and matrix regulation, J. Surg. Res. 84(2) (1999) 127-133.

[23] C. Dionne, G. Crumley, F. Bellot, J. Kaplow, G. Searfoss, M. Ruta, W. Burgess, M. Jaye, J. Schlessinger, Cloning and expression of two distinct high-affinity receptors cross-reacting with acidic and basic fibroblast growth factors, EMBO J. 9(9) (1990) 2685-2692.

[24] H. Kim, Assignment of the human basic fibroblast growth factor gene FGF2 to chromosome 4 band q26 by radiation hybrid mapping, Cytogenet. Genome Res. 83(1-2) (1998) 73.

[25]] H.-X. Shi, C. Lin, B.-B. Lin, Z.-G. Wang, H.-Y. Zhang, F.-Z. Wu, Y. Cheng, L.-J. Xiang, D.-J. Guo, X. Luo, The anti-scar effects of basic fibroblast growth factor on the wound repair in vitro and in vivo, PLoS ONE 8 (4) (2013).

[26] S.J. Coleman, C. Bruce, A.-M. Chioni, H.M. Kocher, R.P. Grose, The ins and outs of fibroblast growth factor receptor signalling, Clin. Sci. 127(4) (2014) 217-231.

[27] M. Hannink, D.J. Donoghue, Structure and function of platelet-derived growth factor (PDGF) and related proteins, Biochim Biophys Acta. 989(1) (1989) 1-10.

[28] C.-H. Heldin, Structural and functional studies on platelet-derived growth factor, EMBO J. 11(12) (1992) 4251-4259.

[29] S. Robins, V. Kumar, C.R. Abbas Ak, N. Faustto, Robbins and Cotran pathologic basis of of disease, China: Elsevier, 2010.

[30] X.-m. Meng, D.J. Nikolic-Paterson, H.Y. Lan, TGF-β: the master regulator of fibrosis, Nat. Rev. Nephrol. 12(6) (2016) 325.

[31] J. Massagué, TGFβ signalling in context, Nat. Rev. Mol. Cell Biol. 13(10) (2012) 616-630.

[32] A. Nakao, M. Afrakhte, A. Morn, T. Nakayama, J.L. Christian, R. Heuchel, S. Itoh, M. Kawabata, N.-E. Heldin, C.-H. Heldin, Identification of Smad7, a TGFβ-inducible antagonist of TGF-β signalling, Nature 389(6651) (1997) 631-635.

[33] A.G. Gilman, M.I. Simon, H.R. Bourne, B.A. Harris, R. Long, E.M. Ross, J.T. Stull, R. Taussig, A.P. Arkin, M.H. Cobb, Overview of the alliance for cellular signaling, Nature 420(6916) (2002) 703–706.

[34] J.J. Letterio, A.B. Roberts, Regulation of immune responses by TGF-β, Annu. Rev. Immunol. 16(1) (1998) 137-161.

[35] J. Massagué, Q. Xi, TGF-β control of stem cell differentiation genes, FEBS Lett. 586(14) (2012) 1953-1958.

[36] M.O. Li, R.A. Flavell, TGF-β: a master of all T cell trades, Cell 134(3) (2008) 392-404.

[37] R.C. Harris, E. Chung, R.J. Coffey, EGF receptor ligands, The EGF Receptor Family, Exp Cell Res. 284(1) (2003) 2-13.

[38] G. Carpenter, S. Cohen, Epidermal growth factor, J. Biol. Chem. 265(14) (1990) 7709-7712.

[39] S. Venturi, M. Venturi, Iodine in evolution of salivary glands and in oral health, Nutr. Health 20(2) (2009) 119-134.

[40] M.D. HOLLENBERG, H. GREGORY, Epidermal growth factor-urogastrone: biological activity and receptor binding of derivatives, Mol. Pharmacol. 17(3) (1980) 314-320.

[41] R.S. Herbst, Review of epidermal growth factor receptor biology, Int. J. Radiat. Oncol. Biol. Phys. 59(2) (2004) S21-S26.

[42] S. Rotolo, S. Ceccarelli, F. Romano, L. Frati, C. Marchese, A. Angeloni, Silencing of keratinocyte growth factor receptor restores 5-fluorouracil and tamoxifen efficacy on responsive cancer cells, PLOS ONE 3(6) (2008) e2528.

[43] S. Jhamb, V.N. Vangaveti, U.H. Malabu, Genetic and molecular basis of diabetic foot ulcers: clinical review, J. Tissue Viability 25(4) (2016) 229-236.

[44] A.J. Singer, R.A. Clark, Cutaneous wound healing, N. Engl. J. Med. 341(10) (1999) 738-746.

[45] S. Frank, B. Stallmeyer, H. Kämpfer, N. Kolb, J. Pfeilschifter, Leptin enhances wound re-epithelialization and constitutes a direct function of leptin in skin repair, J. Clin. Investig. 106(4) (2000) 501-509.

[46] S.P. Teixeira, R.M. Domingues, M. Shevchuk, M.E. Gomes, N.A. Peppas, R.L. Reis, Biomaterials for sequestration of growth factors and modulation of cell behavior, Adv. Funct. Mater. (2020) 1909011.

[47] M.J. Escámez, M. García, F. Larcher, A. Meana, E. Muñoz, J.L. Jorcano, M. Del Río, An in vivo model of wound healing in genetically modified skin-humanized mice, J. Investig. Dermatol. 123(6) (2004) 1182-1191.

[48] S.P. Teixeira, R.M. Domingues, M. Shevchuk, M.E. Gomes, N.A. Peppas, R.L. Reis, Biomaterials for sequestration of growth factors and modulation of cell behavior, Adv. Funct. Mater. (2020) 1909011.

[49] S. Noel, C. Fortier, F. Murschel, A. Belzil, G. Gaudet, M. Jolicoeur, G. De Crescenzo, Co-immobilization of adhesive peptides and VEGF within a dextran-based coating for vascular applications, Acta Biomater. 37 (2016) 69-82.

[50] J.D. Weaver, D.M. Headen, J. Aquart, C.T. Johnson, L.D. Shea, H. Shirwan, A.J. García, Vasculogenic hydrogel enhances islet survival, engraftment, and function in leading extrahepatic sites, Sci. Adv 3(6) (2017) e1700184.

[51] H.J. Lee, G.M. Fernandes-Cunha, I. Putra, W.-G. Koh, D. Myung, Tethering growth factors to collagen surfaces using copper-free click chemistry: surface characterization and in vitro biological response, ACS Appl. Mater. Interfaces 9(28) (2017) 23389-23399.

[52] W.L. Murphy, M.C. Peters, D.H. Kohn, D.J. Mooney, Sustained release of vascular endothelial growth factor from mineralized poly (lactide-co-glycolide) scaffolds for tissue engineering, Biomaterials 21(24) (2000) 2521-2527.

[53] I. Freeman, S. Cohen, The influence of the sequential delivery of angiogenic factors from affinity-binding alginate scaffolds on vascularization, Biomaterials 30(11) (2009) 2122-2131.

[54] T. Kobayashi, M. Mizuta, N. Hiwatashi, Y. Kishimoto, T. Nakamura, S.-i. Kanemaru, S. Hirano, Drug delivery system of basic fibroblast growth factor using gelatin hydrogel for restoration of acute vocal fold scar, Auris Nasus Larynx 44(1) (2017) 86-92.

[55] J.W. Choi, Y.S. Kim, J.K. Park, E.H. Song, J.H. Park, M.S. Kim, Y.S. Shin, C.H. Kim, Controlled Release of Hepatocyte Growth Factor from MPEG‐b‐(PCL‐ran PLLA) Diblock Copolymer for Improved Vocal Fold Regeneration, Macromol. Biosci. 17(2) (2017) 1600163.

[56] J. Sharon, D. Puleo, Immobilization of glycoproteins, such as VEGF, on biodegradable substrates, Acta Biomater. 4(4) (2008) 1016-1023.

[57] J.M. Grasman, R.L. Page, G.D. Pins, Design of an in vitro model of cell recruitment for skeletal muscle regeneration using hepatocyte growth factor-loaded fibrin microthreads, Tissue Eng. Part A 23(15-16) (2017) 773-783.

[58] S. Gnavi, L. di Blasio, C. Tonda-Turo, A. Mancardi, L. Primo, G. Ciardelli, G. Gambarotta, S. Geuna, I. Perroteau, Gelatin-based hydrogel for vascular endothelial growth factor release in peripheral nerve tissue engineering, J Tissue Eng Regen Med 11(2) (2017) 459-470.

[59] J. Kim, B. Lin, S. Kim, B. Choi, D. Evseenko, M. Lee, TGF-β1 conjugated chitosan collagen hydrogels induce chondrogenic differentiation of human synovium-derived stem cells, J. Biol. Eng. 9(1) (2015) 1-11.

[60] M.M. Martino, P.S. Briquez, E. Güç, F. Tortelli, W.W. Kilarski, S. Metzger, J.J. Rice, G.A. Kuhn, R. Müller, M.A. Swartz, Growth factors engineered for super-affinity to the extracellular matrix enhance tissue healing, Science 343(6173) (2014) 885-888.

[61] Q. Li, Y. Niu, H. Diao, L. Wang, X. Chen, Y. Wang, L. Dong, C. Wang, In situ sequestration of endogenous PDGF-BB with an ECM-mimetic sponge for accelerated wound healing, Biomaterials 148 (2017) 54-68.

[62] Y. Feng, Q. Li, D. Wu, Y. Niu, C. Yang, L. Dong, C. Wang, A macrophage-activating, injectable hydrogel to sequester endogenous growth factors for in situ angiogenesis, Biomaterials 134 (2017) 128-142.

Chapter 3

Fabrication and Characterization of PG nanofibrous dressings

3.1 Introduction

ECM is a dense fibrous network of proteins and GAGs that regulates cell behaviour and function. [1, 2] Development of ECM mimicking scaffolds is a key challenge in tissue engineering. [3] With the emergence of nanofabrication techniques like electrospinning, researchers have been able to mimic the ECM structurally as the scaffolds produce using this technique are fibrous with fibers mostly in the Nano-sized range. [4] Electrospinning as a facile and universal fiber forming technique has enabled the fabrication of variety of biomaterials into micro/nanometre-diameter fibers, including synthetic polymers, [5, 6] proteins, [7, 8] and lipids.

Scaffolds fabrication involves not only the structural similarity with ECM but also the biochemical characteristics of the developed scaffold. For the electrospun polymeric nanofibrous scaffolds, it is well known that both synthetic as well as natural polymers have their own pros and cons if electrospun individually, since the scaffolds can either possess good biocompatibility (in case of natural polymers) or can possess good structural stability (in case of synthetic polymers). Incorporating both structural as well as biochemical characteristics involves various surface modification techniques which require relatively complicated derivatization chemistry or time-consuming processing techniques for attaining the desirable surface characteristics. [9, 10]

Recently, a compound spinneret composed of two coaxial capillaries was developed for simultaneously co-electrospinning two distinctly different polymer solutions into core-shell fibers.[11-13] This coaxial electrospinning technique has the potential of producing highly porous and fibrous scaffolds with tailored surface properties by co-processing two materials through a facile one-step procedure.[14] The ability of co-electrospinning technique to produce fibers in core-shell morphology has enabled the researchers to exploit the mechanical stability of synthetic polymers as well as biocompatibility of natural polymers to develop structurally stable porous scaffolds with tuned surface characteristics. Various ECM proteins (gelatin, collagen) in combination with synthetic polymers (PCL, PLLA, PLGA) have been successfully co-electrospun to develop core-shell nanofibers in numerous researches. [12, 15, 16].

In this chapter, co-electrospinning nanofabrication technique was employed for fabrication of PG nanofibrous membranes and to characterize such membranes as ideal wound dressings. Biodegradable core-shell fibrous scaffolds (PG), with PCL forming the core of the fibers and gelatin being deposited on the surface of the PCL fibers, were prepared by coaxial electrospinning. PCL was used as the model material to form the core structure of the fibers because of its good mechanical properties and low biodegradability. [17] Gelatin was selected as the model material for deposition on the PCL fibers to form the shell structure because gelatin is well known to promote cell adhesion and proliferation, as it is an ECM component [18-22]. Also considering the application as wound dressing, gelatin in nanofibrous form is known to reduce inflammatory levels by coaxing macrophages towards an anti-inflammatory phenotype [23]. Also, gelatin is proven to have chemotactic effect on migratory fibroblasts [24].

These fabricated PG nanofibrous dressings were extensively characterized for their application as wound dressing. Compared to monofilament PCL or gelatin nanofibrous dressings, our PG nanofibrous dressings were superior and were coupled with the benefits of both PCL and gelatin monofilament nanofibrous dressings, which individually lacked in some or other aspect.

3.2 Materials and method

3.2.1 Co-axial electrospinning of PG nanofibrous patches

Coaxial needle which consists of two stainless steel needles placed concentrically one inside the other (Inner needle 20G and outer needle 14G) was employed for the co-electrospinning process (Figure 3-1a, b). 7% w/v PCL (Mn 80000, Sigma Aldrich Corporation, USA) and 8% w/v Gelatin type A (Sigma Aldrich Corporation, USA) in 2,2,2 Trifluoroethanol (TFE, 99.5% pure, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) were chosen as core and shell materials, respectively. Electrospinning was done at an applied voltage of 15kV using a high voltage amplifier (Seiko Co. LTD., Tokyo, Japan) using a horizontal setup (Figure 3-1c). The two polymers were fed individually using two programmable syringe pumps (YMC Co., LTD. Tokyo, Japan) at a feed rate of 0.4 ml/h for PCL and 0.6 ml/h for Gelatin, respectively. The fibers were collected on metallic collector placed 12 cm from needle tip. The fibers were removed from collector, air-dried for 24 h and were cut into circular discs. Monofilament PCL and gelatin nanofibers were also electrospun for comparison.

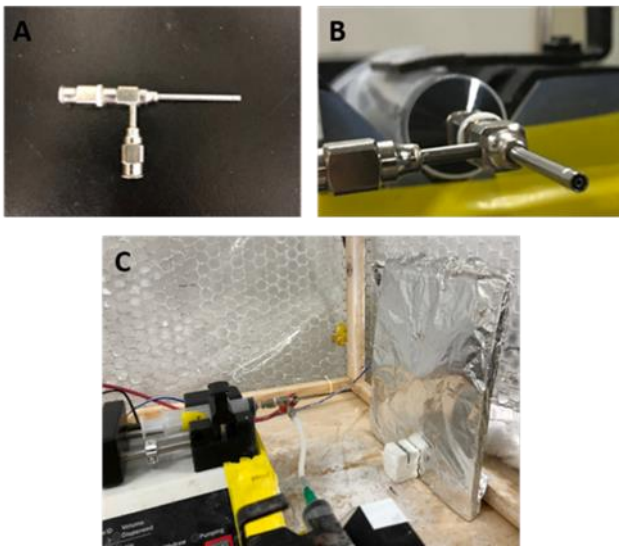


Figure 3-1. Coaxial electrospinning setup. (A) Custom design coaxial needle. Side view. (B) Cross-section view. (C) Horizontal electrospinning setup used in our research.

3.2.2 Crosslinking of PG nanofibrous mats

The crosslinking process was carried out by placing the scaffolds in a sealed desiccator containing a glutaraldehyde solution (25% w/v, Wako chemicals, Tokyo, Japan). The nanofibrous membranes were crosslinked in the glutaraldehyde vapor at room temperature for 1 h. [25]

3.2.3 Physico-chemical characterization

Morphology of nanofibers were observed using scanning electron microscopy (SEM, Hitachi SU3500) at an accelerating voltage of 15 kV. SEM images were utilized for measuring nanofiber diameter distribution, using an image analysis software (ImageJ, NIH, Bethesda, MD, USA).

The core-shell structure of the electrospun PG nanofibers was examined by transmission electron microscopy (TEM, JEOL JEM-2100). For TEM observation, fibers were directly deposited on copper grids and vacuum dried for 48 h before observation.

The PG nanofibers were also characterized using attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy (JASCO FT/IR 620, Tokyo, Japan) over a range of 400-4000 cm-1.

The strength of the PG nanofibers was tested on a tensile and compression testing machine (LTS-50N-S100, Minebea Co., LTD. Nagano, Japan) at a rate of 10 mm/min. The samples were cut following the ASTM D3039 standard and the experiments were performed in triplicates.

The swelling behaviour of PG nanofibers was studied in PBS (pH 7.4). For this study, dry nanofibers were accurately weighed (W0), immersed in PBS and incubated at 37 ℃. At defined time intervals, the nanofibers were withdrawn from the solution and weighed (Wt). The increase in the weight of the nanofibers was measured until saturation was reached. The percentage swelling of the nanofibers was calculated using the following equation:

Percentage swelling = [(Wt -W0)/W0] *100

where, W0 and Wt represent the dry and the wet weights of the nanofibers, respectively.

For degradation analysis, PG nanofiber samples (n=3) were immersed in PBS of pH 7.4 at 37 ℃ under mild stirring and weighed at 7- and 14-days post immersion. The samples were freeze-dried for 24 h before weighing. Degradations of pure PCL and pure gelatin nanofibers were also compared.

Surface wettability of nanofibers were evaluated by calculating water contact angle at room temperature using Drop Shape Analyzer (DSA 100 KRUSS). WVTR values of the

nanofibers were determined according to ASTM E96-95 standard, which measures the weight lost to water vapor transfer.

3.2.4 In-vitro evaluation

Mouse fibroblast cell line L929 from Riken cell bank (Tsukuba, Japan) were cultured in DMEM supplemented with 10% FBS and antibiotics at 37 ℃ under a humidified atmosphere (5% CO_2, 95% air). Passages 2–10 were used for the following experiments and cell culture media was changed every other day. Cells were seeded at density of 5000 cells/cm^2 on three nanofiber samples (PG, Gel and PCL).

Cell adhesion and cytotoxic effects of three scaffolds (PG, Gel and PCL) were observed using cell staining after 48 h of seeding. Cells were stained with Calcein-AM, a highly lipophilic dye; that can interact with cytosolic esterase in viable cells and produce green fluorescence. The cell proliferation was monitored after 1, 3, and 5 days by MTT assay. The mechanism behind this assay is the metabolic reduction of 3-(4,5-dimethylthiozol-2-yl)-2, 5-diphenyltetrazo-lium bromide (MTT) by viable cells.

To evaluate the cytoskeleton organization of the cells on the nanofiber samples, double staining was performed. After 5 days of cell seeding, cells on each of the nanofiber samples were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS for 15 min. Thereafter samples were washed with PBS twice and were incubated with actin green 488 reagent (Thermo Fisher, Yokohama, Japan) for 30 min. After 30 min of incubation, samples were washed with PBS twice and were incubated with diamidino-2-phenylindole (DAPI) (Thermo Fisher, Yokohama, Japan) for 2 min. followed by PBS wash. Washed samples were then suspended in PBS for observation under fluorescent microscope (Olympus IX2-SP, Tokyo, Japan).

3.2.5 Statistical analysis

Results are presented as mean ± standard deviation. The difference between the two groups was evaluated using a two-tailed Student's t-test. *$p<0.05$ was considered as significant; **$p<0.01$ was considered highly significant.

3.3 Results and discussion

3.3.1 Evaluation of morphology

3.3.1.1 Effect of polymer concentration on nanofiber morphology and core-shell structure

With our optimized parameters, we were able to obtain bead-free uniform fibers. The SEM image (Figure 3-2; SEM panel) shows the morphology of fibers obtained using different concentrations of core and shell polymer. As evidenced from the SEM micrograph (Figure 3-2; SEM panel), an increase in polymer concentration of either the core or shell resulted in production of fibers in the micrometer range. However, when concentrations of both polymers were reduced (7% PCL and 8% gelatin; Figure 3-2; SEM panel), we were able to obtain bead free fibers in the nanometer range.

The TEM image (Figure 3-2; TEM panel) clearly indicates the formation of the core-shell structure irrespective of polymer concentration, with the dark component inside the composite being a structural support of PCL. Such a structure resembles a "coating" of gelatin onto individual PCL nanofibers.

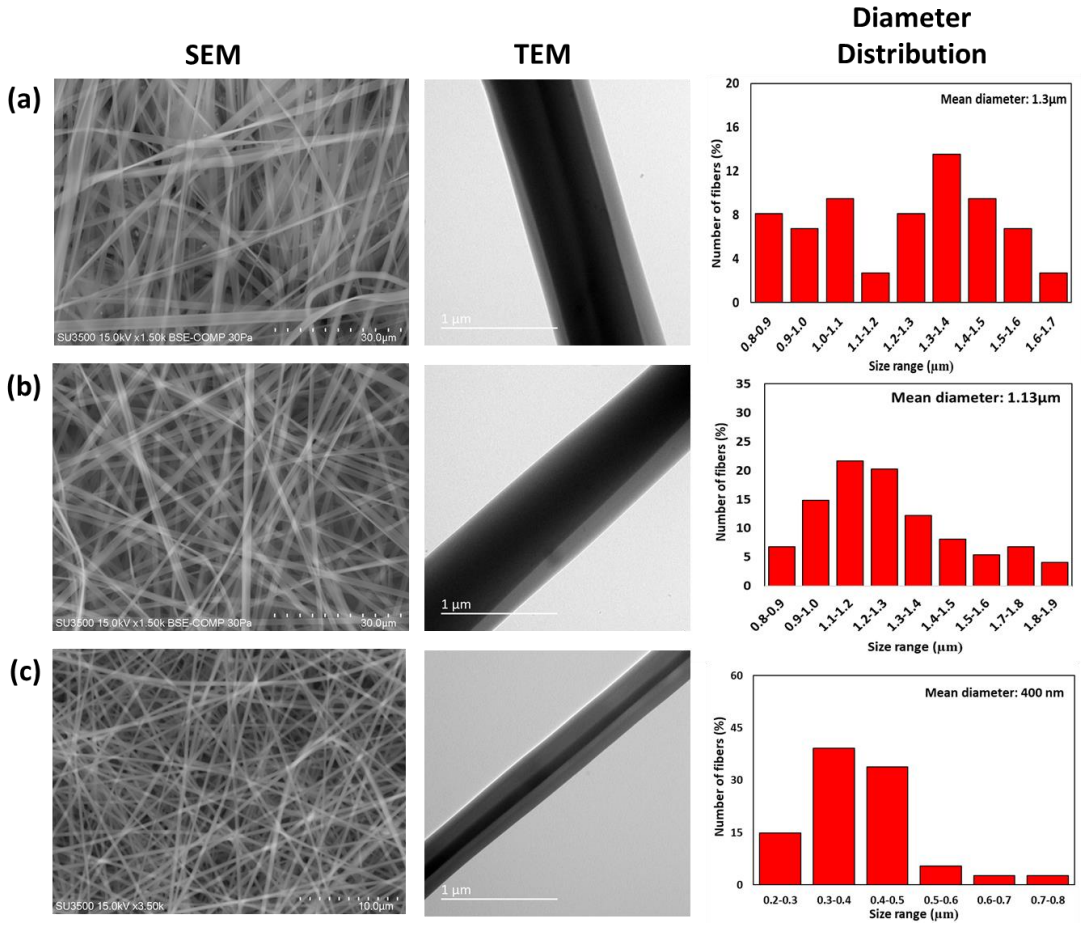


Figure 3-2: SEM and TEM micrograph of PCL/gelatin co-spun nanofabrics corresponding to different concentration of PCL and gelatin. **(a)** 10% PCL and 10% gelatin; **(b)** 10% gelatin and 8 % PCL; **(c)** 8% gelatin and 7% PCL. Feed rate used 0.6 ml/hr. for gelatin, 0.4 ml/hr. for PCL. *Scale bars: SEM-30 µm; TEM-1µm.*

3.3.1.2 Comparison with monofilament PCL and gelatin nanofibers

With our optimized parameters, we were able to obtain bead free uniform fibers. The SEM image shows the fiber morphology of co-spun nanofibers shown in figure 3-3a, clearly depicted that the fiber structure and arrangement of PG nanofibers looked similar to its counterpart monofilament gelatin and PCL nanofibers produced using conventional electrospinning technique. Also, nanofibers produced using both conventional and

coaxial techniques show a normal distribution and average diameters of about 370 nm (Figure 3-3b).

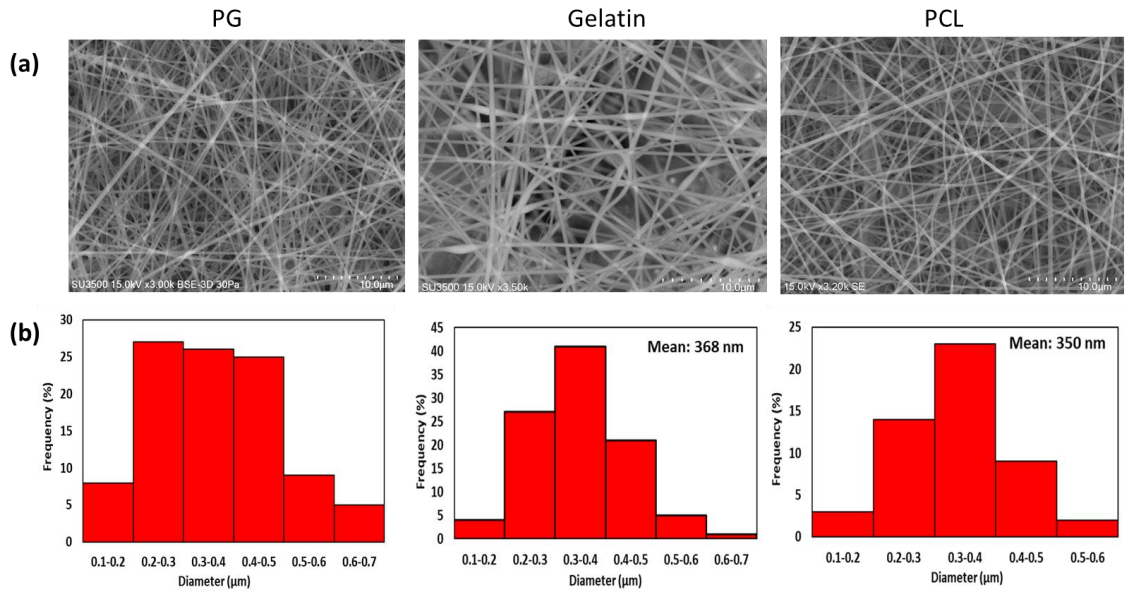


Figure 3-3: SEM micrograph of PG, gelatin and PCL nanofibers. (a) Showing structural similarity of PG nanofibers with their counterpart monofilament Gelatin and PCL nanofibers; (b) Diameter distribution.

3.3.2 Physico-chemical characterization

The FTIR and tensile tests for PG nanofibers are shown in Figure 3-4. The characteristic peak that are solely isolated to gelatin are described as: 3443 cm^{-1} (N-H stretching of amide bonds), 1640 cm^{-1} (C-O stretching) and 1543 cm^{-1} (N-H bending). For PCL, the characteristic peaks solely isolated to PCL are described as: 1740 cm^{-1} (C=O stretching), 1240 cm^{-1} (C-O-C stretching). The peaks that are common to both PCL and gelatin are described as: 2949 cm^{-1} (asymmetric CH2 stretching) and 2865 cm^{-1} (symmetric CH2 stretching) [26]. These peaks were analyzed to confirm the presence of both polymers. Presence of all above characteristics peak in FTIR spectrum of PG nanofibers before

washing gelatin shell confirms presence of both PCL and gelatin in the electrospun nanofabrics (Figure 3-4). Moreover, no peak shifts were observed in the spectra of PG nanofibers indicating no interactions between core and shell polymers. Also, the spectrum looked more dominated by gelatin characteristic peaks showing intense magnitude. However, when gelatin shell was removed, the spectrum showed increment in magnitude in the peaks solely isolated to PCL (1240 cm^{-1}, 1727 cm^{-1}). Alterations in peak intensities for PCL before and after washing can be another probable confirmation of core-shell structure.

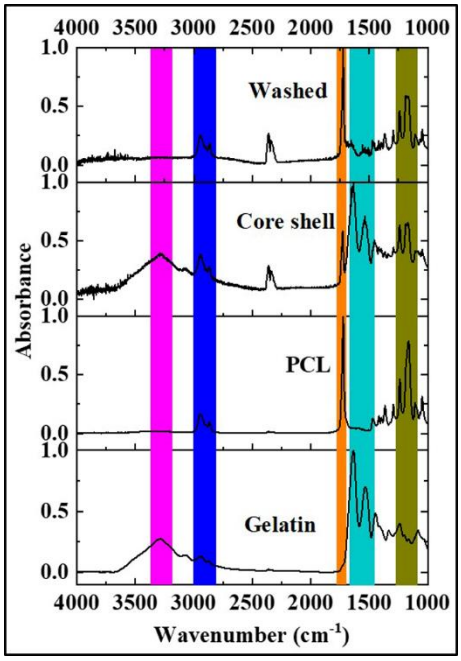


Figure 3-4: FTIR spectrum of Gelatin, PCL and PG nanofibers.

Mechanical properties of PG nanofibers are shown in Figure 3-5 (a, b). An improvement in mechanical properties can be observed (Strain at fracture 45%, Ultimate Yield Strength 5 MPa) compared to monofilament gelatin nanofibers which were inferior in terms of mechanical properties

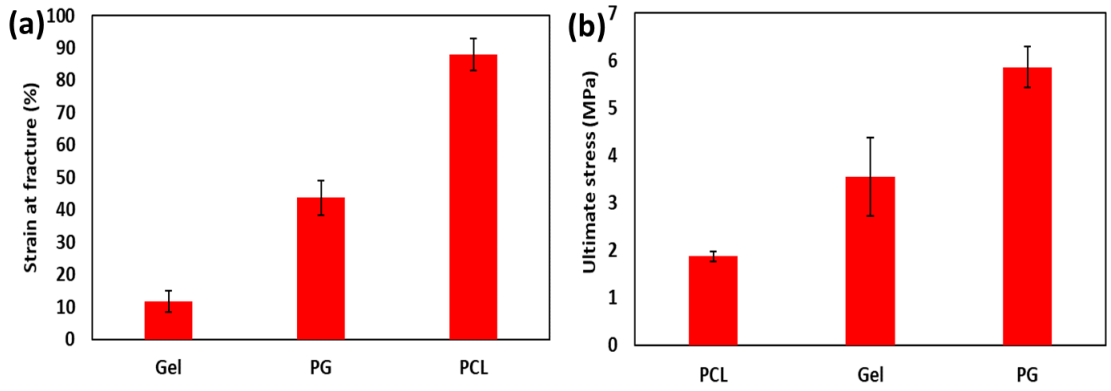


Figure 3-5: Mechanical properties of PG nanofibers compared to their counterpart monofilament gelatin and PCL nanofibers.

This effect of improvement in mechanical properties are further demonstrated in Figure 3-6, which clearly shows that PG nanofibers were more elastic and can retain their structure which makes their handling easier which ensures their easy application at wound bed compared to their gelatin counterparts.

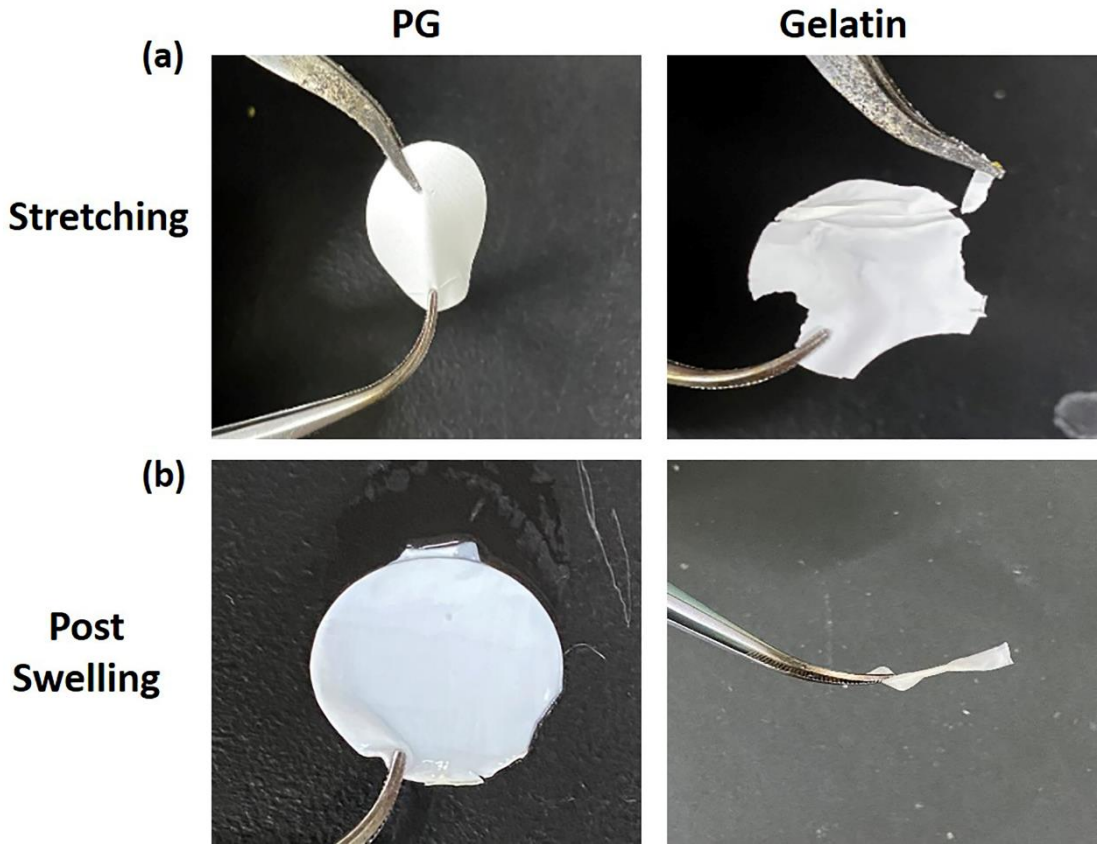


Figure 3-6: PG nanofibers shows good mechanical properties (a) and can maintain their structure post swelling (b). In comparison gelatin nanofibers were fragile and cannot maintain their structure post swelling which makes their handling difficult.

Swelling behaviour of PG nanofibers are shown in Figure 3-7a. Good swelling ability is essential for wound dressings to absorb wound exudates upon application [27]. In terms of swelling behaviour PG nanofibers were comparable to their counterpart monofilament gelatin nanofibers and shows good swelling abilities. In contrary PCL nanofibers were inferior and lacks when it comes to swelling ability. Additionally, in terms of degradation rate, improvements can be observed (Figure 3-7b). PG nanofibers shows negligible weight loss (14-18%) till 14 days and were comparable to PCL nanofibers in terms of their degradability. On the other hand, gelatin nanofibers show high degradation with 70-

80% weight loss within 14 days. Overall, PG nanofibers were ideal in terms of mechanical properties, swelling abilities and degradability and were coupled with the benefits of both PCL and gelatin monofilament counterparts, which were inferior in some or other context.

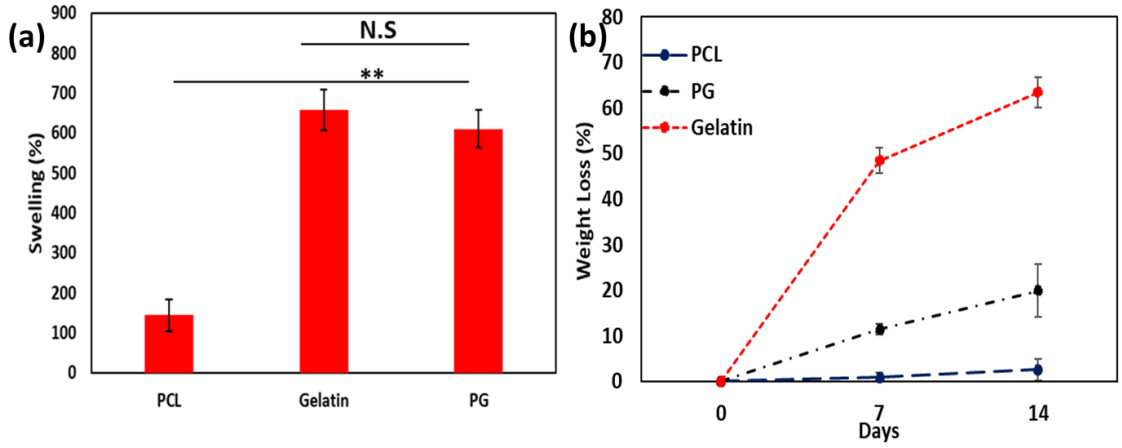


Figure 3-7: Swelling and degradation analysis of PG nanofibers compared to their monofilament counterparts. (a) Swelling ability; (b) Degradation

WVTR and surface wettability of PG nanofiber are shown in Figure 3-8a and b. Ideal WVTR value of dressing should be between 2000-2500 g/m^2/day in order to prevent the dehydration and accumulation of extra exudates [28]. PG nanofibers shows WVTR value in the range 2467±243 g/m^2/day (Figure 3-8a) revealing that the produced scaffolds were ideal wound dressings in terms of their WVTR values. The WVTR values of monofilament PCL and gelatin nanofibers were also in ideal range (Gelatin: 2657±509 g/m^2/day and PCL: 2500±300 g/m^2/day). Contact angle measurements for nanofiber samples are shown in Figure 3-8b. In terms of surface wettability PG and gelatin nanofibers demonstrated outstanding hydrophilicity with no significant difference in contact angle measurement (PG: 20 ± 6º, Gel: 19.72 ± 2º) indicating their suitability as wound dressings. In contrast PCL monofilament nanofibers were poor in terms of surface wettability (PCL: 95 ± 3º).

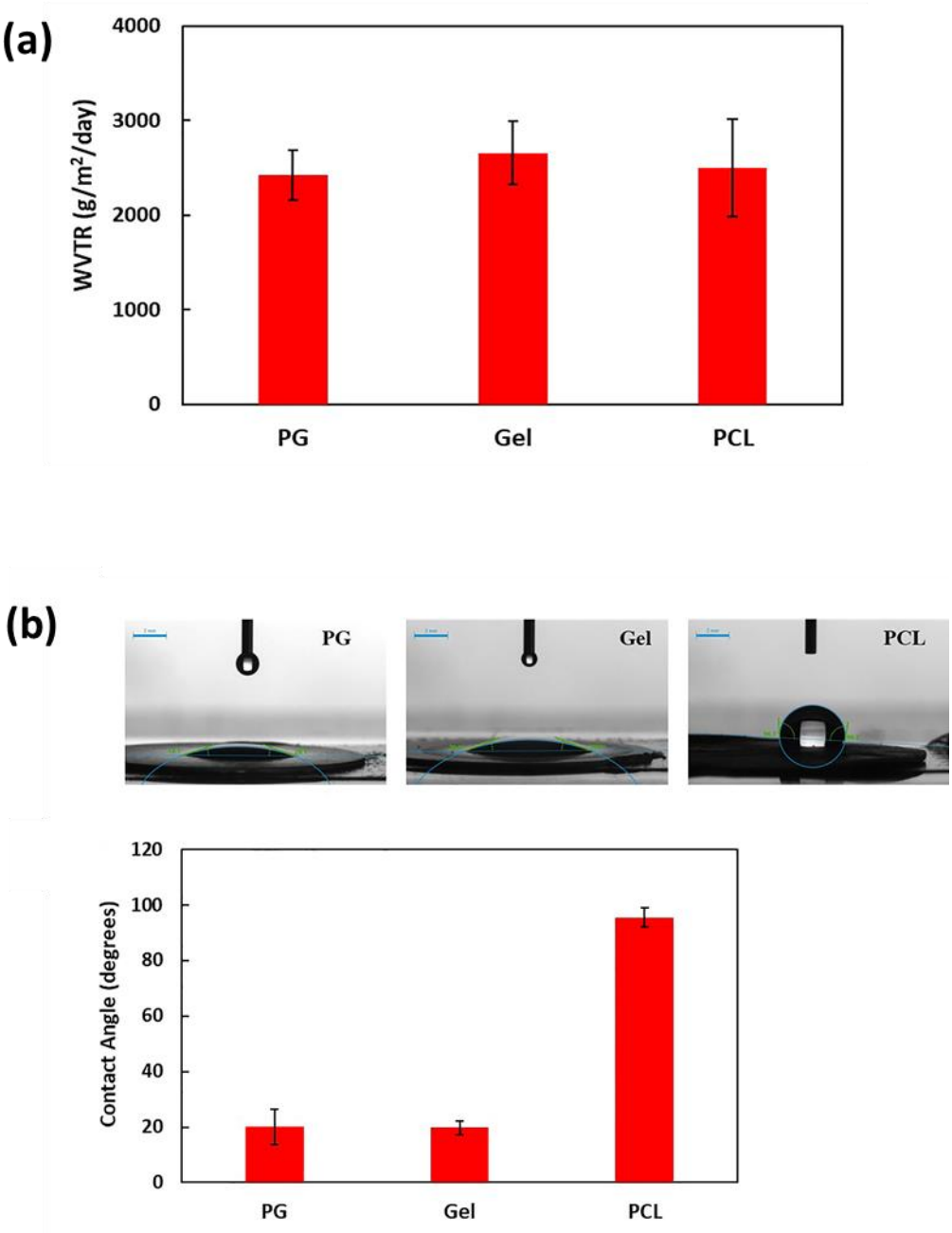


Figure 3-8: WVTR and surface wettability of PG nanofibers compared to their monofilament Gelatin and PCL counterparts. (a) WVTR; (b) Contact angle measurement.

3.3.3 Biocompatibility evaluation of PG nanofibers

Figure 3-9 shows calcein-AM staining of adhered cells. Cells adhered to each of the nanofiber samples. All the samples exhibited strong green florescence, indicating good live cell density on each scaffold, indicating no cytotoxicity of fabricated nanofabrics.

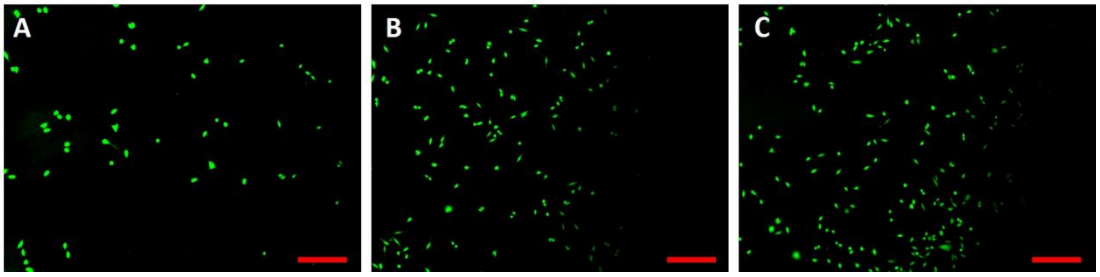


Figure 3-9. Calcein-AM staining of different nanofiber samples. (A) PCL, (B) Gelatin and (C) PG. Scale bars 100 µm.

Figure 3-10 shows the growth of cells on the three nanofiber samples. Cells proliferated in each scaffold till day 5 as seen from WST-8 data of cell proliferation. PG and gelatin nanofibers showed no significant difference in cell growth. However, the growth of cells on PCL nanofabrics was slower. This could be due to lack of integrin binding sites in PCL nanofabrics.

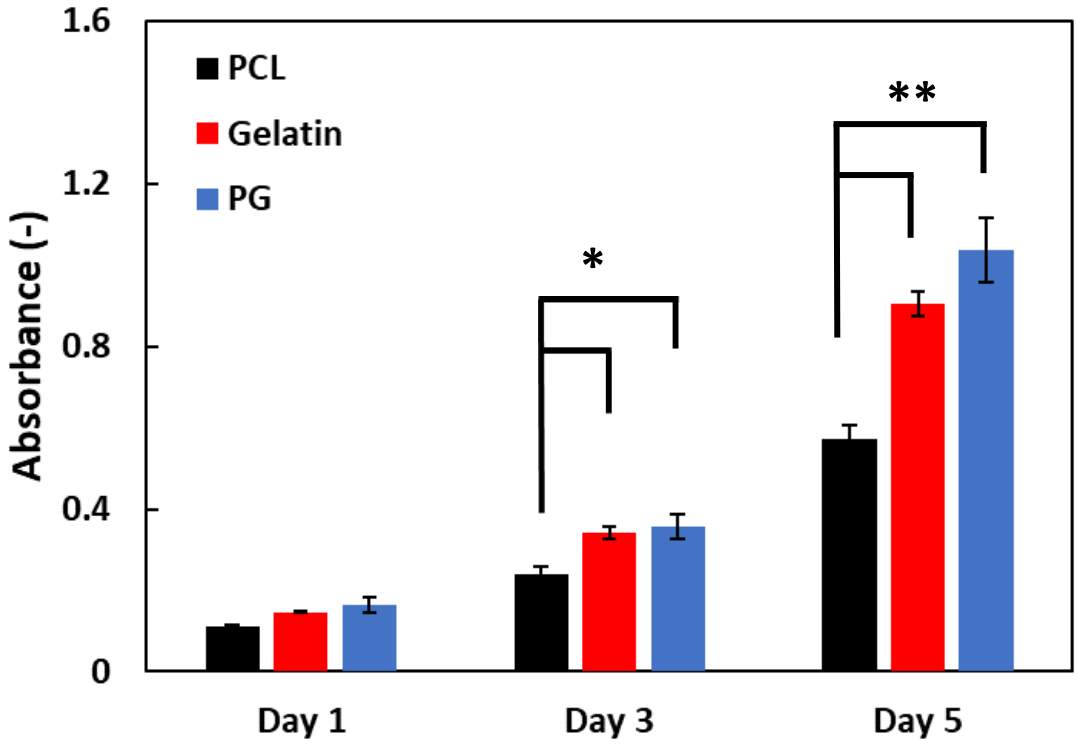


Figure 3-10: WST-8 cell proliferation assay. *p<0.05

The results of cytoskeleton staining are shown in figure 3-11. As seen from figure 11, cells cultured on monofilament PCL nanofibers showed least spreading morphology. The spreading was significant higher on monofilament gelatin nanofibers and highest on PG nanofibers, indicating that PG nanofibers were superior in promoting proper cell growth and spreading compared to monofilament PCL or gelatin nanofibers. The reason for this superiority of PG nanofabrics over monofilament counterparts is their closeness to skin in terms of mechanical characteristics (Tensile strain: 48%, close to skin elasticity which ranges from 50-75%) as well as ECM mimicking nature. In contrast, though gelatin nanofabrics were ECM mimicking, they showed fragile nature which ensures a mismatch with skin mechanical properties. On the other hand, though PCL nanofabrics showed good mechanical characteristics, they lack integrin binding sites.

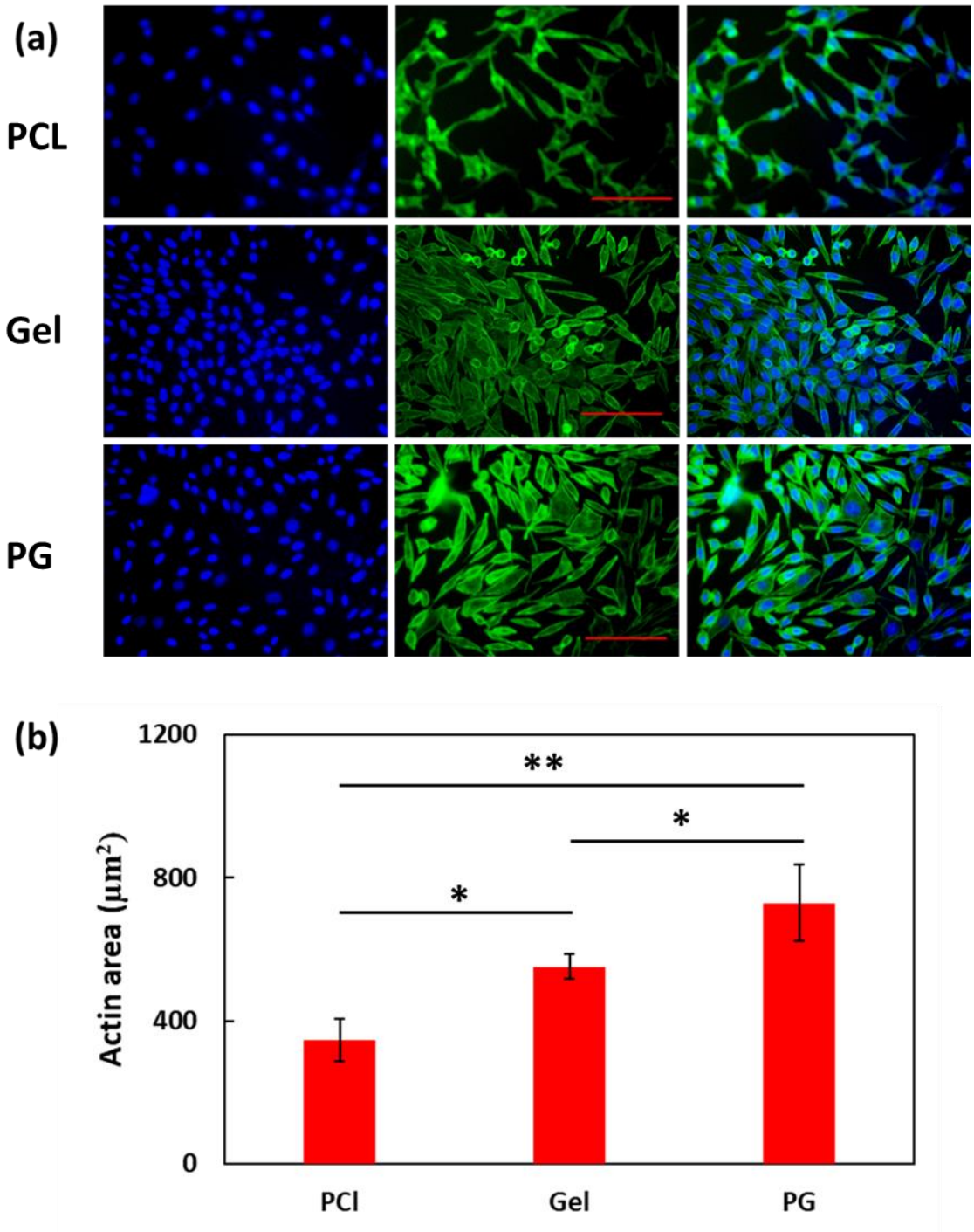


Figure 3-11: Cytoskeleton arrangement of cells cultured on different nanofiber samples. (a) Actin/DAPI staining showing cytoskeleton arrangement; (b) Actin area. *p<0.05; **p<0.01. *Scale bars: 200 µm.*

3.5 Discussion

Tissue engineering aims to develop scaffolds which mimics the in vivo environment structurally as well as functionally to promote good cell-scaffold interaction [29]. Because of which electrospinning technique has been widely used by researchers to develop scaffolds due to structural integrity between native ECM and electrospun scaffolds [30, 31]. The effectiveness of nanofibrous structure in promoting cell growth has been demonstrated by numerous researchers. Various natural ECM proteins have been successfully electrospun to nanofibrous structure to promote bioactivity as well as functionality, however electrospun natural polymeric scaffolds often possess poor mechanical properties that limits their capability to be used as scaffolds. Electrospun synthetic polymers though possess tuned mechanical characteristics, often lacks bioactivity. Incorporation of both mechanical as well as bioactivity to scaffolds is difficult to achieve using electrospinning technique. Because of which, hybridisation techniques like co-electrospinning plays an important role wherein both the characteristics can be incorporated onto the scaffolds through a simple one step procedure.

Numerous researches have shown the effectiveness of co-electrospinning in incorporating bioactivity as well as structural stability to the scaffolds. Zhang et al. demonstrated the effectiveness of PCL/collagen co-spun scaffolds over surface modified scaffolds. [32] Also Merkle et al. demonstrated the efficiency of this technique to develop tissue engineered artificial blood vessel over blend polymeric scaffolds. [33]

Our objective was to employ co-electrospinning technique, owing to its advantage over other scaffold fabrication technique and to evaluate the effectiveness of these co-axial electrospun nanofibrous membranes as ideal wound dressings. The first and foremost

important pre-requisite of an ideal dressing is its balance between biocompatibility and mechanical properties. As reported previously [34], elasticity of 50-75% is ideal to mimic the mechanical properties of native skin and to promote proper migration of fibroblasts, that are principle cells in skin regeneration. By employing co-axial electrospinning technique, we were able to obtain nanofibrous membranes that showed elasticity close to this value (Tensile strain: 48%), without compromising with its ECM mimicking nature due to presence of ECM component, gelatin, on its surface. These nanometre range gelatin coated PCL nanofibrils can closely mimic skin structurally as well as in terms of its mechanical properties [35].

Apart from having good mechanical properties, an ideal dressing should also have excellent surface wettability as well as ideal WVTR values. This ensures proper cell migration as well as prevents the dehydration and accumulation of extra exudates at wound site. Our PG nanofibrous membranes showed excellent surface wettability as well as ideal WVTR [28] values that ensures their suitability to be employed as wound dressings.

Lastly, dressings should not have any cytotoxic response as well as should support proper cellular growth. Our PG nanofabrics were not cytotoxic to the cultured fibroblast and supported proper cellular growth and spreading, ensuring that the nanofabrics showed suitability in terms of this parameter. Overall, our PG nanofabrics were endowed with all the capabilities of ideal wound dressings.

3.6 Conclusion

PG nanofibrous scaffolds with improved biochemical as well as mechanical properties was fabricated in our research. The scaffold possesses good mechanical strength as well

as promoted proper cell growth and spreading compared to monofilament nanofibrous scaffolds fabricated using traditional techniques. Also, the fabricated nanofabrics showed outstanding surface wettability and ideal WVTR values. Owing to these characteristics, such fabricated nanofabrics can show tremendous application to be applied as wound dressings.

References

[1]. Griffith, L. G.; Naughton, G., Tissue engineering--current challenges and expanding opportunities, Science 295(5557) (2002) 1009-1014.

[2]. Bosman, F. T.; Stamenkovic, I., Functional structure and composition of the extracellular matrix, J. Pathol. 200(4) (2003) 423-428.

[3]. Shin, H.; Jo, S.; Mikos, A. G., Biomimetic materials for tissue engineering, Biomaterials 24(24) (2003) 4353-4364.

[4]. Lee, C. H.; Shin, H. J.; Cho, I. H.; Kang, Y.-M.; Kim, I. A.; Park, K.-D.; Shin, J.-W., Nanofiber alignment and direction of mechanical strain affect the ECM production of human ACL fibroblast, Biomaterials 26(11) (2005) 1261-1270.

[5]. Li, W. J.; Laurencin, C. T.; Caterson, E. J.; Tuan, R. S.; Ko, F. K., Electrospun nanofibrous structure: a novel scaffold for tissue engineering, J. Biomed. Mater. Res. 60(4) (2002) 613-621.

[6]. Yang, F.; Murugan, R.; Wang, S.; Ramakrishna, S., Electrospinning of nano/micro scale poly (L-lactic acid) aligned fibers and their potential in neural tissue engineering. Biomaterials 26(15) (2005) 2603-2610.

[7]. Matthews, J. A.; Wnek, G. E.; Simpson, D. G.; Bowlin, G. L., Electrospinning of collagen nanofibers, Biomacromolecules 3(2) (2002) 232-238.

[8]. Sell, S. A.; Wolfe, P. S.; Garg, K.; McCool, J. M.; Rodriguez, I. A.; Bowlin, G. L., The use of natural polymers in tissue engineering: a focus on electrospun extracellular matrix analogues, Polymers 2(4) (2010) 522-553.

[9]. Cronin, E. M.; Thurmond, F. A.; Bassel-Duby, R.; Williams, R. S.; Wright, W. E.; Nelson, K. D.; Garner, H. R., Protein-coated poly (L-lactic acid) fibers provide a substrate for differentiation of human skeletal muscle cells, J. Biomed. Mater. Res. 69(3) (2004) 373-381.

[10]. Hu, Y.; Winn, S. R.; Krajbich, I.; Hollinger, J. O., Porous polymer scaffolds surface-modified with arginine-glycine-aspartic acid enhance bone cell attachment and differentiation in vitro, J. Biomed. Mater. Res. 64(3) (2003) 583-590.

[11]. Jiang, H.; Hu, Y.; Li, Y.; Zhao, P.; Zhu, K.; Chen, W., A facile technique to prepare biodegradable coaxial electrospun nanofibers for controlled release of bioactive agents, J. Control. Release 108(2-3) (2005) 237-243.

[12]. Zhang, Y.; Wang, X.; Feng, Y.; Li, J.; Lim, C.; Ramakrishna, S., Coaxial electrospinning of (fluorescein isothiocyanate-conjugated bovine serum albumin)-encapsulated poly (ε-caprolactone) nanofibers for sustained release, Biomacromolecules 7(4) (2006) 1049-1057.

[13]. Yarin, A., Coaxial electrospinning and emulsion electrospinning of core–shell fibers, Polym. Adv. Technol. 22(3) (2011) 310-317.

[14]. Zhang, Y.; Huang, Z.-M.; Xu, X.; Lim, C. T.; Ramakrishna, S., Preparation of core−shell structured PCL-r-gelatin bi-component nanofibers by coaxial electrospinning, Chem. Mater. 16(18) (2004) 3406-3409.

[15]. Ma, Z.; He, W.; Yong, T.; Ramakrishna, S., Grafting of gelatin on electrospun poly (caprolactone) nanofibers to improve endothelial cell spreading and proliferation and to control cell orientation, Tissue Eng. 11(7-8) (2005) 1149-1158.

[16]. Zhang, Y.; Su, B.; Venugopal, J.; Ramakrishna, S.; Lim, C., Biomimetic and bioactive nanofibrous scaffolds from electrospun composite nanofibers, Int J Nanomedicine. 2(4) (2007) 623-638.

[17]. Kim, G. H., Electrospun PCL nanofibers with anisotropic mechanical properties as a biomedical scaffold, Biomed. Mater. 3(2) (2008) 025010.

[18]. Ki, C. S.; Baek, D. H.; Gang, K. D.; Lee, K. H.; Um, I. C.; Park, Y. H., Characterization of gelatin nanofiber prepared from gelatin–formic acid solution, Polymer 46(14) (2005) 5094-5102.

[19]. Huang, Z.-M.; Zhang, Y.; Ramakrishna, S.; Lim, C., Electrospinning and mechanical characterization of gelatin nanofibers, Polymer 45(15) (2004) 5361-5368.

[20]. Z. Ma, W. He, T. Yong, S. Ramakrishna, Grafting of gelatin on electrospun poly (caprolactone) nanofibers to improve endothelial cell spreading and proliferation and to control cell orientation, Tissue Eng. 11(7-8) (2005) 1149-1158.

[21]. J.-P. Chen, C.-H. Su, Surface modification of electrospun PLLA nanofibers by plasma treatment and cationized gelatin immobilization for cartilage tissue engineering, Acta Biomater. 7(1) (2011) 234-243.

[22]. K. Park, Y.M. Ju, J.S. Son, K.-D. Ahn, D.K. Han, Surface modification of biodegradable electrospun nanofiber scaffolds and their interaction with fibroblasts, J. Biomater. Sci. 18(4) (2007) 369-382.

[23]. R.-X. Wu, C. Ma, Y. Liang, F.-M. Chen, X. Liu, ECM-mimicking nanofibrous matrix coaxes macrophages toward an anti-inflammatory phenotype: Cellular behaviors and transcriptome analysis, Appl. Mater. Today 18 (2020) 100508.

[24]. A.E. Postlethwaite, J.M. Seyer, A.H. Kang, Chemotactic attraction of human fibroblasts to type I, II, and III collagens and collagen-derived peptides, Proc. Natl. Acad. Sci. U.S.A. 75(2) (1978) 871-875.

[25]. S. Bhowmick, S. Rother, H. Zimmermann, P.S. Lee, S. Moeller, M. Schnabelrauch, V. Koul, R. Jordan, V. Hintze, D. Scharnweber, Biomimetic electrospun scaffolds from main extracellular matrix components for skin tissue engineering application–The role of chondroitin sulfate and sulfated hyaluronan, Mater. Sci. Eng. C 79 (2017) 15-22.

[26]. M.S. Kim, I. Jun, Y.M. Shin, W. Jang, S.I. Kim, H. Shin, The development of genipin‐crosslinked poly (caprolactone)(PCL)/gelatin nanofibers for tissue engineering applications, Macromol. Biosci. 10(1) (2010) 91-100.

[27]. Z. Wu, Y. Hong, Combination of the Silver–Ethylene Interaction and 3D Printing To Develop Antibacterial Superporous Hydrogels for Wound Management, ACS Appl. Mater. Interfaces 11(37) (2019) 33734-33747.

[28]. R. Xu, H. Xia, W. He, Z. Li, J. Zhao, B. Liu, Y. Wang, Q. Lei, Y. Kong, Y. Bai, Controlled water vapor transmission rate promotes wound-healing via wound re-epithelialization and contraction enhancement, Sci. Rep. 6 (2016) 24596.

[29]. W. Jia, M. Li, L. Kang, G. Gu, Z. Guo, Z. Chen, Fabrication and comprehensive characterization of biomimetic extracellular matrix electrospun scaffold for vascular tissue engineering applications, J. Mater. Sci. 54(15) (2019) 10871-10883.

[30]. S. Chahal, A. Kumar, F.S.J. Hussian, Development of biomimetic electrospun polymeric biomaterials for bone tissue engineering. A review, J. Biomater. Sci. 30(14) (2019) 1308-1355.

[31]. E. Türker, Ü.H. Yildiz, A.A. Yildiz, Biomimetic hybrid scaffold consisting of co-electrospun collagen and PLLCL for 3D cell culture, Int. J. Biol. Macromol. 139 (2019) 1054-1062.

[32]. Y. Zhang, J. Venugopal, Z.-M. Huang, C. Lim, S. Ramakrishna, Characterization of the surface biocompatibility of the electrospun PCL-collagen nanofibers using fibroblasts, Biomacromolecules 6(5) (2005) 2583-2589.

[33]. V.M. Merkle, L. Zeng, M.J. Slepian, X. Wu, Core‐shell nanofibers: Integrating the bioactivity of gelatin and the mechanical property of polyvinyl alcohol, Biopolymers 101(4) (2014) 336-346.

[34]. J. He, Y. Liang, M. Shi, B. Guo, Anti-oxidant electroactive and antibacterial nanofibrous wound dressings based on poly (ε-caprolactone)/quaternized chitosan-graft-polyaniline for full-thickness skin wound healing, Chem. Eng. J. 385 (2020) 123464.

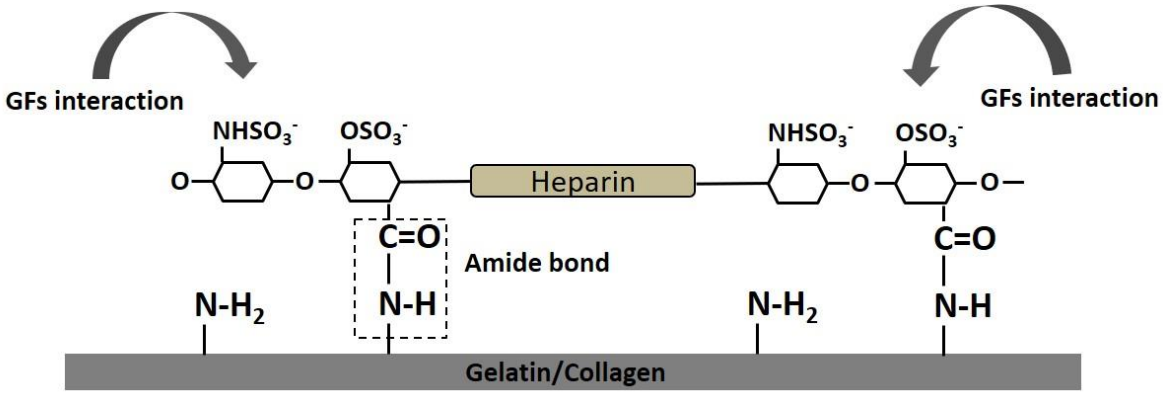
[35]. Buehler, M. J., Nature designs tough collagen: explaining the nanostructure of collagen fibrils, Proc. Natl. Acad. Sci. U.S.A. 103(33) (2006) 12285-12290.

Chapter 4

Inclusion of heparin to PG nanofabrics and evaluation of their exogenous and endogenous growth factors sequestration ability

4.1 Introduction

GFs based therapeutics has been the most researched among all protein-based therapeutics. Especially when it comes to wound healing applications, GFs have been the most researched proteins owing to their involvement in all phases of healing. Among most available biomaterial-based approach in delivering GFs, functionalization or inclusion of a biomaterial with heparin is the most widely used technique, due to ability of heparin in interacting with several GFs via electrostatic interactions [1-4]. Heparin has strong affinity for numerous GFs; importantly for pro-regenerative GFs, VEGF and PDGF; the concentration of which drastically increases at the site of injury [5-7]. Also though heparin is unlikely to form a matrix on its own, it can be covalently conjugated as functional cues in a 3-D ECM mimicking scaffold to sequester these locally produced GFs (Scheme 1), thanks to its structural and functional similarity with heparan sulphate, which is the primary GAG molecule in the mammalian cell matrices that stabilizes and empower several endogenous GFs [8-10].



Scheme 4-1. Mechanism showing heparin conjugation to ECM via amide bond formation. The negatively charged heparin molecule can then interact with several GFs via electrostatic forces.

Though heparin has been widely incorporated as a GF sequestering moiety in tissue engineering scaffolds, to sequester and release a range of GFs and has been well reported in numerous researches, none of the researches conducted so far has demonstrated the efficacy of heparin conjugated biomaterials to sequester locally produced endogenous GFs at injury site and utilize the endogenous GFs power for obtaining regeneration with minimum dependency on exogenous proteins that have concerns related to safety and efficacy. Therefore, in this chapter, an attempt has been made to evaluate the ability of PG nanofabrics fabricated in previous chapter in sequestering GFs from exogenous and endogenous sources through functionalization with heparin. PG nanofabrics functionalized with heparin via EDC/NHS coupling, called as Hep-PG, were extensively evaluated for their exogenous and endogenous GFs activity. From our results, our fabricated Hep-PG nanofabrics showed tremendous potential in sequestering GFs from both exogenous and endogenous sources.

4.2 Materials and method

4.2.1 Fabrication of nanofabrics

Coaxial needle which consists of two stainless steel needles placed concentrically one inside the other (Inner needle 20G and outer needle 14G) was employed for the co-electrospinning process. 7% w/v PCL (Mn 80000, Sigma Aldrich Corporation, USA) and 8% w/v Gelatin type A (Sigma Aldrich Corporation, USA) in 2,2,2 Trifluoroethanol (TFE, 99.5% pure, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) were chosen as core and shell materials, respectively. Electrospinning was done at an applied voltage of 15kV using a high voltage amplifier (Seiko Co. LTD., Tokyo, Japan). The two polymers were fed individually using two programmable syringe pumps (YMC Co., LTD. Tokyo, Japan) at a feed rate of 0.4 ml/h for PCL and 0.6 ml/h for Gelatin, respectively. The fibers were collected on metallic collector placed 12 cm from needle tip. The fibers were removed from collector, air-dried for 24 h and were cut into circular discs. These discs were then immobilized with heparin and were evaluated for their exogenous and endogenous GFs sequestration ability.

4.2.2 Heparin immobilization and quantification

Heparin was conjugated to PG scaffolds using the procedure below [11, 12]. PG nanofibers were soaked in activated solution of heparin sodium (1 mg/ml; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) containing 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; 1 mg/ml; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and N-Hydroxy succinimide (NHS; 0.6 mg/ml; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) in 0.05 M MES buffer (800 µl/sample). Nanofibers without heparin conjugation (PG) and conjugated to heparin without use of EDC/NHS (Hep-PG-E) were taken as control. The conjugation reaction was carried out

for 2 h at room temperature under mild stirring. After 2 h, the supernatants were collected, and immobilized density was calculated using TB assay by evaluating the unreacted heparin in the supernatant using a previously generated standard curve. Samples were also stained using TB to obtain a visual confirmation [13,14].

4.2.3 Evaluation of exogenous and endogenous sequestering capability of Hep-PG nanofibers

To evaluate the exogenous GF sequestering capability of Hep-PG nanofibers, bFGF was taken as a representative example. For the immobilization of bFGF (Recombinant human bFGF; R&D Systems; USA), Hep-PG nanofibers were equilibrated in PBS overnight at 4 ℃. All scaffolds were then blocked with 1% bovine serum albumin in PBS solution for 1h to minimize the passive adsorption of bFGF. Blocked membranes were then treated with different concentration of bFGF (10 ng/ml, 50 ng/ml in PBS; 200 µl/scaffold) for 3 h at 4 ℃. Post 3 h treatment, the supernatants were collected and the sequestered bFGF was calculated by assaying the amount of unbounded bFGF in the supernatant using the bFGF ELISA kit (R&D Systems; USA) following the manufacturer's protocol [15, 16]. Non heparin functionalized membranes (PG) were taken as control for comparison.

To evaluate the endogenous GFs sequestering capability of Hep-PG nanofibers, nanofiber discs were directly placed at the wound site for 3 days (n=4). Non heparin functionalized membranes (PG) were taken as control for comparison. Post 3 days, nanofibers were recovered from the wound site, washed gently using PBS 3 times and were soaked in collagenase solution (1 mg/ml; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) for 3 h at 37 ℃ to completely degrade the gelatin. Post 3 h incubation at 37 ℃, the supernatant post degradation was collected and amount of one of the pro-regenerative

endogenous GF, VEGF, in the supernatant was checked using VEGF ELISA (R&D Systems; USA) following manufacturer's protocol.

4.2.4 In vitro cell culture study

4.2.4.1 Preparation of electrospun scaffolds

For cell culture studies, nanofibers were electrospun onto glass cover slips (13 mm diameter), that can completely cover the base of 24-well plate using the above-mentioned electrospinning parameters. The coverslips were air-dried for 24 h in fume-hood and were crosslinked for 1 h in desiccator. The crosslinked membranes (PG) were further air dried for 24 h and were then conjugated to heparin. Heparin conjugated nanofibers (Hep-PG) were then sterilized using subsequent washings with 70% ethanol and PBS. These sterilized membranes were then treated with a solution of bFGF for 3 h at 4 ℃ (100 ng/ml, 50 ng/ml). Post 3 h of treatment, membranes were washed with PBS to remove physically adsorbed bFGF. Hep-PG nanofibers without bFGF treatment were also prepared as control groups.

4.2.4.2 Cell culture

Mouse fibroblast cell line L929 from Riken cell bank (Tsukuba, Japan) was cultured in DMEM supplemented with 10% FBS and antibiotics (1% Penicillin/Streptomycin) at 37 ℃ under a humidified atmosphere of 5% CO_2/95% air [17]. Cells at passages 2-10 were used for this experiment. Cells were seeded on nanofiber samples with and without bFGF treatment. Cells were cultured for five days in media containing 5% FBS to analyze the effect of sequestered bFGF on cell growth.

4.2.4.3 Cell viability and cytoskeleton arrangement

To analyze the live/ dead cell density on nanofibrous membranes, cells at 2 and 4 days of culture were stained using Calcein-Am (Thermo Fisher Scientific; USA)/ Propidium Iodide (PI, Thermo Fisher Scientific; USA) double staining [18]. Stained samples were observed using fluorescence microscope (Olympus IX 73). For cytoskeleton staining, cells at Day 5 of culture were fixed using 10% formalin for 30 min. Fixed samples were blocked using 1% BSA solution and were double-stained for actin using Alexa Fluor 594 Phalloidin (Thermo Fischer Scientific, USA) for 30 min, and for nuclei using 4′,6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific, USA) for 5 min. The stained samples were then observed using a confocal laser microscope (TCS SP8; Leica Microsystems, Wetzlar, Germany).

4.2.5 Statistical analysis

Results are presented as mean ± standard deviation. The difference between the two groups was evaluated using a two-tailed Student's t-test. *$p<0.05$ was considered as significant; **$p<0.01$ was considered highly significant.

4.3 Results and discussion

4.3.1 Fabrication of wound dressings

Figure 4-1a shows the nanofiber sheet withdrawn from collector. The collected nanofiber sheet was air-dried for 24h and were then cut into circular discs of 10mm diameter. These discs were then immobilized with heparin to get the final heparin immobilized wound dressings (Figure 4-1b).

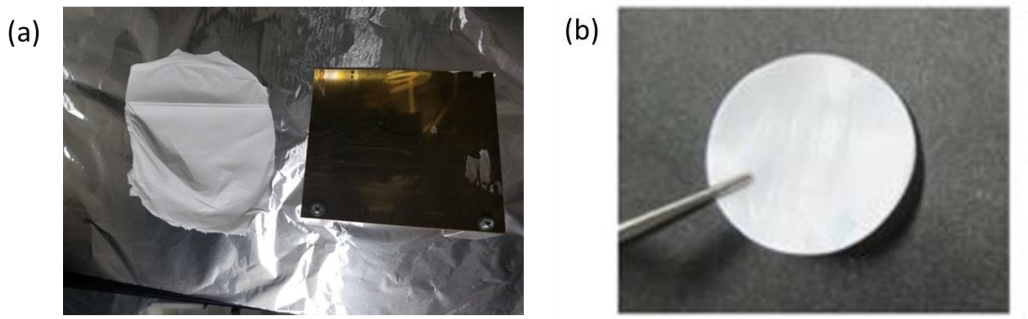


Figure 4-1: (a) Nanofiber sheet removed from metallic collector; (b) Punched heparin functionalized nanofiber discs.

4.3.2 Evaluation of heparin functionalization

The results of TB staining and quantification analysis is shown in Figure 9 (a, b, c). It can be clearly seen from TB staining (Figure 4-2) that under conjugation in presence of EDC/NHS, the scaffolds were completely stained with TB whereas scaffolds conjugated without EDC/NHS (Hep-PG-E) showed almost negligible staining, comparable to the nanofibers with no heparin coupling (PG).

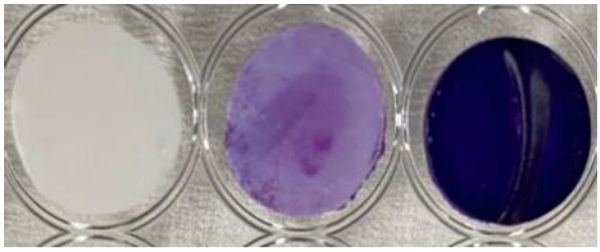


Figure 4-2. Toluidine blue staining for visual observation of heparin coupling. PG represents PCL/gelatin co-spun nanofibers with no heparin conjugation, He-PG-E represents PCL/gelatin co-spun nanofibers wherein heparin conjugation was carried without EDC/NHS and Hep-PG represents PCL/gelatin co-spun nanofibers with EDC/NHS mediated heparin coupling.

The results of quantification are listed in Figure 4-3. Significant higher amount of heparin was conjugated in presence of EDC/NHS (Figure 4-3a; **$p<0.01$; 250 µg/cm^2) with a conjugation efficiency of 50% (Figure 4-3b). In comparison, amount of conjugated heparin without EDC/NHS was low (80 µg/cm^2, Figure 4-3a) with efficiency as low as 17% (Figure 4-3b).

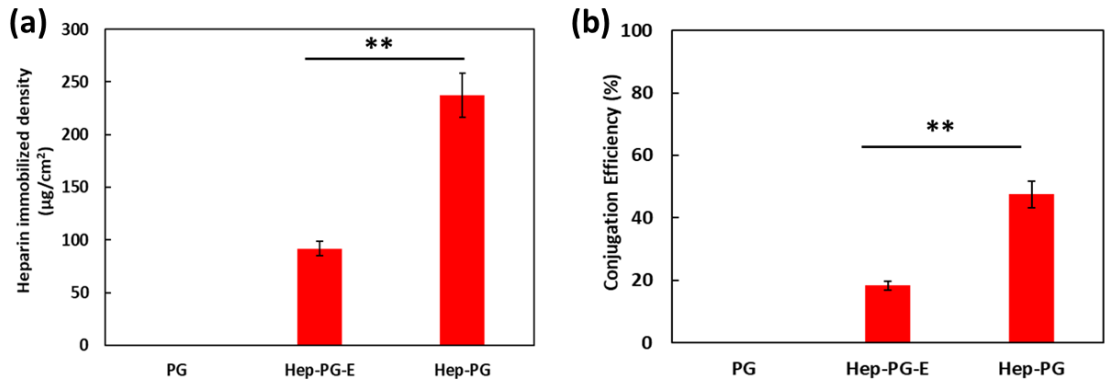


Figure 4-3. Quantification of immobilized heparin. (a) Conjugated amount; (b) Conjugation efficiency. **p<0.01.

Further coupling of heparin to PG nanofabrics was also evaluated through FTIR analysis. FTIR spectrum of PG and Hep-PG nanofibers is shown in Figure 4-4. The characteristic peak that are associated to gelatin are described as: 3443 cm^{-1} (N-H stretching of amide bonds), 1640 cm^{-1} (C-O stretching) and 1543 cm^{-1} (N-H bending). Similarly, the characteristic peaks associated to PCL are described as: 1740 cm^{-1} (C=O stretching), 1240 cm^{-1} (C-O-C stretching), 1160 cm^{-1} (symmetric C–O–C stretching). The peaks that are common to both PCL and gelatin are described as: 2949 cm^{-1} (asymmetric CH2 stretching) and 2865 cm^{-1} (symmetric CH2 stretching). Presence of all above characteristics peak in FTIR spectrum of PG confirms presence of both PCL and gelatin in the electrospun nanofabrics (Figure 4-4). Additionally, when functionalization with heparin was achieved (Hep-PG), the peaks at 1240 cm^{-1} and 1160 cm^{-1} (peaks highlighted in green, Figure 4-4) showed increment in magnitude. These peaks were associated to asymmetric and symmetric stretching vibrations of the O=S=O group in the heparin, which confirms the heparin coupling.

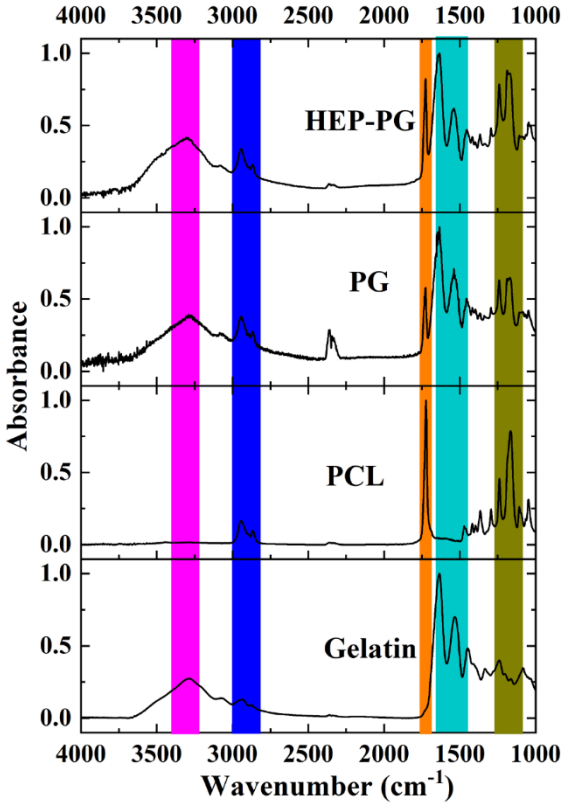


Figure 4-4: FTIR spectrum of PG and Hep-PG nanofabrics. Alterations in peak associated to heparin confirms heparin coupling.

Further the contribution of physical adsorption and chemical conjugated amount in Hep-PG nanofabrics was evaluated. To evaluate the contribution of physically adsorbed heparin, the release profile of heparin from Hep-PG (nanofibers with covalently conjugated heparin) and Hep-PG-E (nanofibers with physically adsorbed heparin) was performed. The results are illustrated in figure 4-5. Complete removal of heparin was observed from nanofibers within 48 h when there was no covalent conjugation, indicating that physically adsorbed heparin was completely removed within 48 h. In case of Hep-PG nanofibers, it was 5-6% heparin that was released within 48 h, with no further release indicating that covalent conjugation of heparin is indispensable to prevent loss of heparin

from dressings. Preventing loss of heparin from dressings is essential as, firstly, heparin should be retained as it is responsible for sequestration of GFs from endogenous source. Secondly, controlled release is important as excess heparin released on wound bed may interfere with clotting cascade. Simply adding heparin to the electrospun nanofabrics or by adding it to the spinning solution may risk in burst release profile. Hence, we conjugated heparin covalently to ensure there is minimum release of heparin from our nanofabrics.

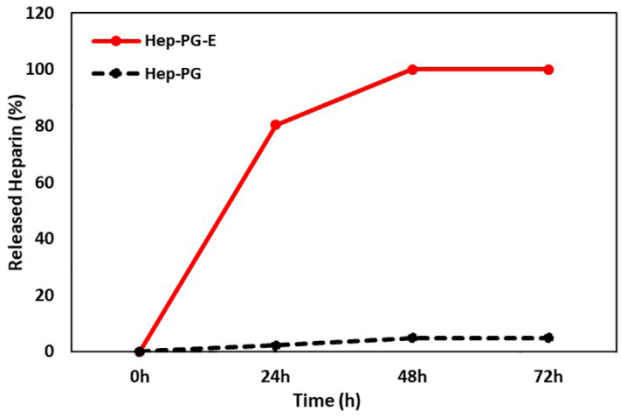


Figure 4-5: Release profile of heparin from nanofibers under different condition of immobilization. Hep-PG represents nanofibers with covalently conjugated heparin; Hep-PG-E represent nanofibers with physically adsorbed heparin.

4.3.3 Evaluation of exogenous bFGF sequestration ability of Hep-PG nanofibers

Ability of Hep-PG nanofibers to sequester exogenous bFGF is shown in Figure 4-6 (a, b). As seen from Figure 4-6a, when nanofibers were functionalized with heparin (Hep-PG), significant higher amount of bFGF was immobilized (approximately 2 ng/cm^2 when added amount was 10 ng/ml and approximately 6 ng/cm^2 when added amount was 50 ng/ml; **$p<0.01$) compared to control groups with no heparin functionalization (PG; approximately 0.6 ng/cm^2 when added amount was 10 ng/ml and approximately 2 ng/cm^2

when added amount was 50 ng/ml). An estimated 82% of the bFGF was incorporated into the heparinized nanofibrous membranes (Hep-PG; Figure 4-6b) and the corresponding degree of incorporation was as low as 30% under non-heparinized condition (PG; Figure 4-6b). These results clearly indicate the effectiveness of heparin molecules to sequester exogenous GFs with high efficiency. Since through functionalization with heparin, we can achieve high efficiency to load exogenous bFGF into our dressings, we believe these loaded GFs based on heparin can be released in a controlled manner once a dressing is applied at the wound site, as heparin based delivery have been well proven to be sustained and controlled.

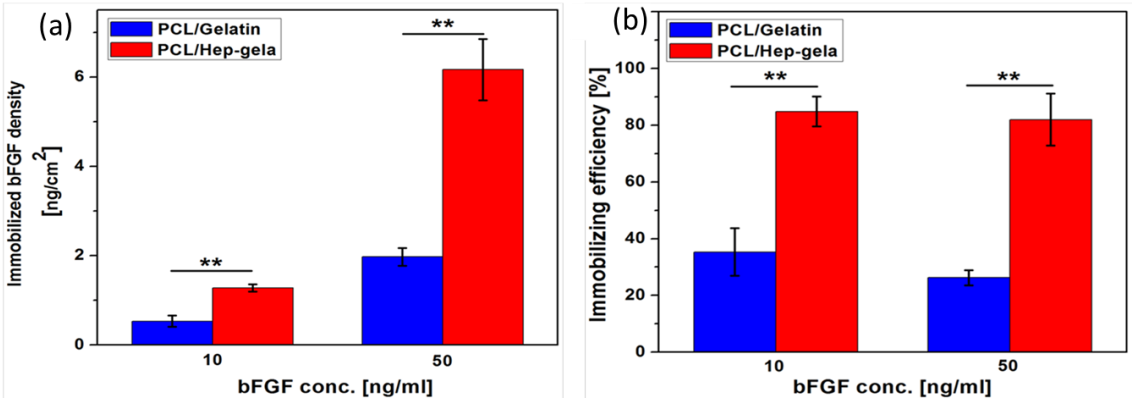


Figure 4-6: Exogenous bFGF sequestration capability of Hep-PG nanofibers. (a) Density of immobilized bFGF; (b) Efficiency of immobilized bFGF. **P<0.01.

4.3.3 Evaluation of endogenous pro-regenerative GF sequestration ability of Hep-PG nanofibers

The endogenous GF sequestering capability of Hep-PG nanofibers is shown in Figure 4-7a. Compared to non-heparin functionalized membranes (PG), Hep-PG nanofibers can sequester 15-fold higher amount of endogenous VEGF indicating the efficiency of heparin molecules in sequestering endogenous pro-regenerative GFs. It is to be noted that

even with preloaded bFGF, the sequestration trend of endogenous VEGF was similar and showed no significant difference (Figure 4-7b), indicating that exogenous loading of bFGF does not affect the endogenous sequestration ability of Hep-PG nanofibers. At this stage, we demonstrated availability of heparin to sequester GFs from endogenous source. Such sequestration is very important, as GFs released at wound sites often undergo early degradation due to protease rich environment. By employing our fabricated dressings, these GFs can be sequestered in earlier stages of healing and can be utilized at later stages of healing, which would have otherwise undergone degradation. This is what previous researches have failed to demonstrate and have utilized GFs only from exogenous sources. We believe our findings can be valuable in the field of wound care and management, towards utilizing power of endogenous GFs and reducing the dependency on exogenous protein that suffer from concerns related to safety and efficacy.

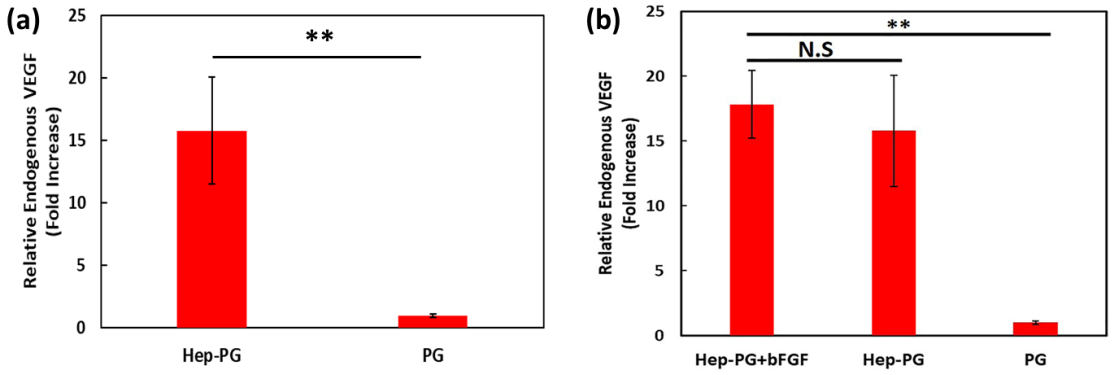


Figure 4-7: Endogenous pro-regenerative GFs sequestration capability of Hep-PG nanofibers demonstrated by using VEGF as a representative example. (a) Sequestration of endogenous VEGF by Hep-PG nanofibers; (b) Sequestration of endogenous VEGF with pre-immobilized exogenous VEGF.

4.3.4 In-vitro evaluation by culturing L929 fibroblast cells

Hep-PG nanofibers can stabilize GFs by sequestering them. To confirm this phenomenon, difference in cellular behaviour were observed by culturing cells (L-929 mouse fibroblast) on Hep-PG nanofibers with and without bFGF treatment, by assaying the cell viability and cytoskeleton arrangement of cells. When Hep-PG nanofibers were treated with solution of bFGF (50 ng/ml, 100 ng/ml), they can maintain higher viability of cultured fibroblast, compared to Hep-PG nanofibers that were not treated with bFGF solution (Figure 4-8).

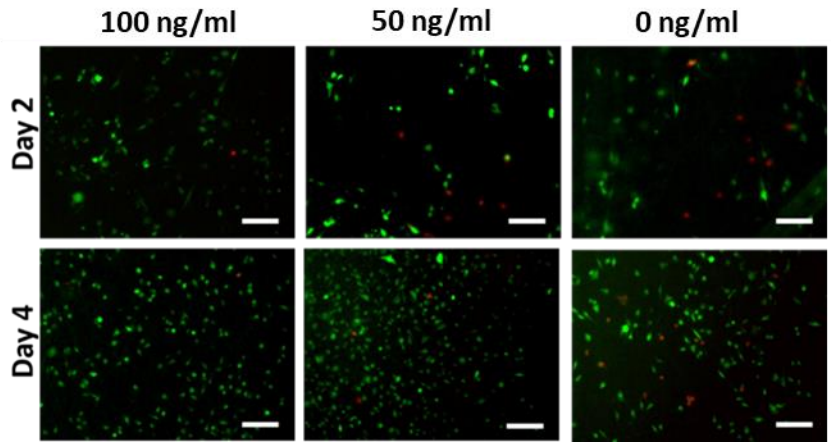


Figure 4-8: Calcein-AM/PI staining of L929 cells cultured on Hep-PG nanofibers treated with different concentration of exogenous bFGF. Green: Live cells; Red: Dead cells. Scale bars: 200 µm.

To further confirm this effect, the cytoskeleton arrangement of cells was observed on Hep-PG nanofibers with and without bFGF treatment. Clear differences in cellular morphology can be seen (Figure 4-9a) with significant higher cell spreading on membranes treated with bFGF (Figure 4-9b). This data confirms their ability to not only

sequester bFGF with high efficiency, but also in stabilizing the activity of bFGF by this mechanism.

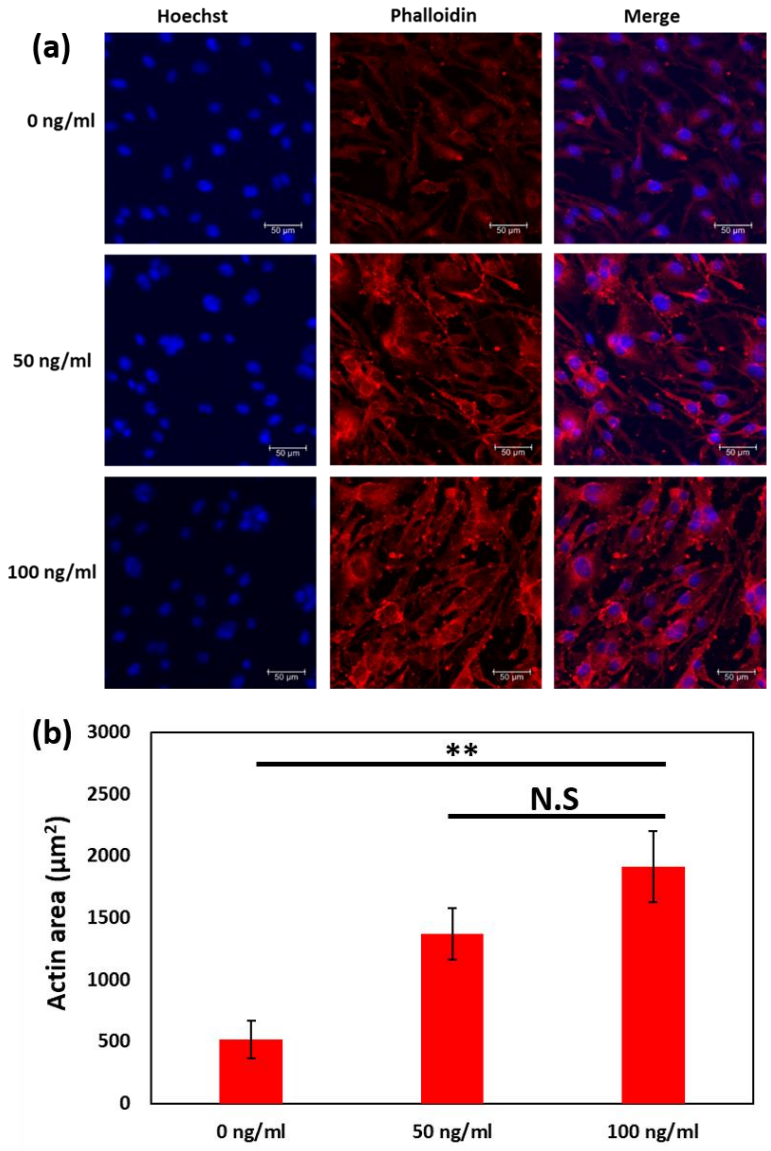


Figure 4-9: Cytoskeleton arrangement of L929 cells cultured on Hep-PG nanofibers immobilized with different concentration of bFGF. (a) Actin/DAPI stained L929 cells; (b) Actin area plot. **p<0.01.

4.4 Discussion

Wound dressings that can stimulate proper tissue regeneration are highly demanded. Over the years, numerous researches have been conducted to fabricate ideal dressings that can promote effective tissue regeneration. Nevertheless, few current biomaterials scaffolds could recapitulate the prominent feature of ECM, to sequester and release GFs which are key meditators in tissue regeneration. It emerges as a pressing need to device a scaffold that can mimic ECM in sequestering the endogenous, pro-regenerative GFs around the site of injury and thereby create a suitable niche to facilitate endogenously driven tissue regeneration.

To achieve such target, we considered three parameters that we believe can be considered prerequisite for fabrication of an ideal dressing. Firstly, to have minimum inflammatory response post transplantation and to facilitate proper cellular migration during the application, the dressing should closely mimic the structure of ECM. Skin is mostly composed of collagen, which have fibril like structure ranging up to few 100 of nanometers [19]. To mimic such structure, nanofibrous dressings were fabricated. Further, to ensure closeness to skin ECM along with proper mechanical properties to allow ease of handling, co-axial electrospinning technique was adopted for dressing fabrication wherein gelatin coated PCL nanofibrous patches with fiber diameter ranging few 100 of nanometers were fabricated. We chose PCL as core component due to its good mechanical properties whereas gelatin was chosen as shell component, to ensure the dressings closely mimic skin ECM, as gelatin is a derivative of collagen. Moreover, gelatin is cost effective and available in abundant compared to collagen. Apart from ECM mimicking nature, our fabricated PG nanofabrics showed excellent mechanical properties

and ideal wettability and WVTR values. All these properties make it effective to be employed as wound dressing as a first prerequisite.

Secondly, the dressings should have the capability to stabilize the endogenous GFs by sequestering them. If biomaterials employed as wound dressings have this property, the therapeutic effect of endogenous GFs can be utilized, and tissue regeneration can be achieved with minimum dependency on exogenous GFs delivery. To endow nanofabrics with endogenous GFs sequestering capability, surface functionalization with heparin was achieved via EDC/NHS coupling, due to its well-known ability in binding and enhancing the function of heparin binding GFs both in-vitro and in-vivo. From our results, we were able to show tremendous capability of Hep-PG nanofabrics in sequestering endogenous pro-regenerative GFs with high efficiency.

Thirdly, apart from being ECM mimicking and having capability to sequester endogenous GFs, the dressings should itself have therapeutic effect to enhance the tissue regeneration. To endow therapeutic capability into our fabricated dressings, we loaded limited amount of exogenous bFGF. bFGF was chosen as a candidate for exogenous delivery due to its well-known property to be involved in almost all phases of healing. Also, bFGF is known for its ability to promote scarless tissue regeneration when administered exogenously and has been clinically proven [20-23].

Our fabricated Hep-PG nanofabrics showed all the above capabilities. Thus, our fabricated biomaterial was coupled with the benefits of an ideal wound dressing plus with therapeutic capability due to its ability to sequester GFs both from exogenous and endogenous sources.

4.5 Conclusion

This chapter demonstrated an ideal way to fabricate wound dressings with minimum dependency on exogenous GFs and utilizing endogenous GFs therapeutic effect for obtaining cost-effective and accelerated tissue regeneration. Hep-PG nanofibers were analyzed for their exogenous and endogenous GF sequestering capability. Taken together, we demonstrated an innovative and feasible biomaterials-based approach to sequester GFs from both exogenous and endogenous sources based on heparin functionalization to biomaterial. We believe our fabricated Hep-PG nanofabrics might promote accelerated tissue regeneration by utilizing the synergistic effect between exogenous bFGF and endogenous GFs sequestration ability.

References

[1] R. Flaumenhaft, D. Moscatelli, D.B. Rifkin, Heparin and heparan sulfate increase the radius of diffusion and action of basic fibroblast growth factor, J. Cell Biol. 111(4) (1990) 1651-1659.

[2] M.H. Hettiaratchi, L. Krishnan, T. Rouse, C. Chou, T.C. McDevitt, R.E. Guldberg, Heparin-mediated delivery of bone morphogenetic protein-2 improves spatial localization of bone regeneration, Sci. Adv. 6(1) (2020) eaay1240.

[3] J. Ishihara, A. Ishihara, K. Fukunaga, K. Sasaki, M.J. White, P.S. Briquez, J.A. Hubbell, Laminin heparin-binding peptides bind to several growth factors and enhance diabetic wound healing, Nat. Commun. 9(1) (2018) 1-14.

[4] S. Kang, J.S. Yoon, J.Y. Lee, H.-J. Kim, K. Park, S.E. Kim, Long-term local PDGF delivery using porous microspheres modified with heparin for tendon healing of rotator cuff tendinitis in a rabbit model, Carbohydr. Polym. 209 (2019) 372-381.

[5] Y. Feng, Q. Li, D. Wu, Y. Niu, C. Yang, L. Dong, C. Wang, A macrophage-activating, injectable hydrogel to sequester endogenous growth factors for in situ angiogenesis, Biomaterials 134 (2017) 128-142.

[6] Q. Li, Y. Niu, H. Diao, L. Wang, X. Chen, Y. Wang, L. Dong, C. Wang, In situ sequestration of endogenous PDGF-BB with an ECM-mimetic sponge for accelerated wound healing, Biomaterials 148 (2017) 54-68.

[7] S.P. Teixeira, R.M. Domingues, M. Shevchuk, M.E. Gomes, N.A. Peppas, R.L. Reis, Biomaterials for sequestration of growth factors and modulation of cell behavior, Adv. Funct. Mater. (2020) 1909011.

[8] M. Qiao, L. Lin, K. Xia, J. Li, X. Zhang, R.J. Linhardt, Recent advances in biotechnology for heparin and heparan sulfate analysis, Talanta (2020) 121270.

[9] F. Zsila, T.n. Juhász, G. Kohut, T.s. Beke-Somfai, Heparin and Heparan Sulfate Binding of the Antiparasitic Drug Imidocarb: Circular Dichroism Spectroscopy, Isothermal Titration Calorimetry, and Computational Studies, J. Phys. Chem. B 122(6) (2018) 1781-1791.

[10] K. Sugahara, H. Kitagawa, Heparin and heparan sulfate biosynthesis, IUBMB life 54(4) (2002) 163-175.

[11] J. Lee, J.J. Yoo, A. Atala, S.J. Lee, The effect of controlled release of PDGF-BB from heparin-conjugated electrospun PCL/gelatin scaffolds on cellular bioactivity and infiltration, Biomaterials 33(28) (2012) 6709-6720.

[12] J. Lee, J. Yoo, A. Atala, S. Lee, Controlled heparin conjugation on electrospun poly (ε-caprolactone)/gelatin fibers for morphology-dependent protein delivery and enhanced cellular affinity, Acta Biomater. 8(7) (2012) 2549-2558.

[13] S. Nakamura, T. Kubo, H. Ijima, Heparin-conjugated gelatin as a growth factor immobilization scaffold, J. Biosci. Bioeng. 115(5) (2013) 562-567.

[14] J.-h. Lee, Y.J. Lee, H.-j. Cho, D.W. Kim, H. Shin, The incorporation of bFGF mediated by heparin into PCL/gelatin composite fiber meshes for guided bone regeneration, Drug Deliv. Transl. Res. 5(2) (2015) 146-159.

[15] S.A. DeLong, J.J. Moon, J.L. West, Covalently immobilized gradients of bFGF on hydrogel scaffolds for directed cell migration, Biomaterials 26(16) (2005) 3227-3234.

[16] O. Jeon, S.-W. Kang, H.-W. Lim, J.H. Chung, B.-S. Kim, Long-term and zero-order release of basic fibroblast growth factor from heparin-conjugated poly (L-lactide-co-glycolide) nanospheres and fibrin gel, Biomaterials 27(8) (2006) 1598-1607.

[17] J. Sun, Z. Dai, Y. Zhao, G.-Q. Chen, In vitro effect of oligo-hydroxyalkanoates on the growth of mouse fibroblast cell line L929, Biomaterials 28(27) (2007) 3896-3903.

[18] D. Chawla, T. Kaur, A. Joshi, N. Singh, 3D bioprinted alginate-gelatin based scaffolds for soft tissue engineering, Int. J. Biol. Macromol. 144 (2020) 560-567.

[19] M.J. Buehler, Nature designs tough collagen: explaining the nanostructure of collagen fibrils, Proc. Natl. Acad. Sci. U.S.A. 103(33) (2006) 12285-12290.

[20] H.-X. Shi, C. Lin, B.-B. Lin, Z.-G. Wang, H.-Y. Zhang, F.-Z. Wu, Y. Cheng, L.-J. Xiang, D.-J. Guo, X. Luo, The anti-scar effects of basic fibroblast growth factor on the wound repair in vitro and in vivo, PLOS ONE 8(4) (2013) e59966.

[21] S. Tiede, N. Ernst, A. Bayat, R. Paus, V. Tronnier, C. Zechel, Basic fibroblast growth factor: a potential new therapeutic tool for the treatment of hypertrophic and keloid scars, Ann. Anat. 191(1) (2009) 33-44.

[22] H.-L. Xu, P.-P. Chen, L.-F. Wang, M.-Q. Tong, Z.-h. Ou, Y.-Z. Zhao, J. Xiao, T.-L. Fu, Skin-permeable liposome improved stability and permeability of bFGF against skin of mice with deep second degree scald to promote hair follicle neogenesis through inhibition of scar formation, Colloids Surf. 172 (2018) 573-585.

[23] A. Joshi, Z. Xu, Y. Ikegami, K. Yoshida, Y. Sakai, A. Joshi, T. Kaur, Y. Nakao, Y.-I. Yamashita, H. Baba, Exploiting synergistic effect of externally loaded bFGF and

endogenous growth factors for accelerated wound healing using heparin functionalized PCL/gelatin co-spun nanofibrous patches, Chem. Eng. J. (2020) 126518.

Chapter 5

In-vivo evaluation of Hep-PG nanofibers with exogenous bFGF loading capability and endogenous growth factors sequestration ability in promoting accelerated tissue regeneration

5.1 Introduction

Skin wound healing involves complex dynamics and a series of multicellular processes as the tissue regenerates [1]. Although the skin tissue can regenerate itself in due course, the healing often takes time and can result in improper tissue regeneration with scarring and lack of skin appendages [2, 3]. This is especially true for chronic wounds, which affects millions of patients and have been associated with an economic cost of USD 20 billion annually [4]. Traditional wound dressing materials are usually insufficient to allow the regeneration of damaged tissues as they only protect the wounded skin from the external environment by providing a temporary barrier [5]. The use of skin grafts in treatments is very costly and does not guarantee the full regain of skin functionalities.

Cost effective treatment involves design of biomaterials for wound healing and tissue regeneration which aims to restore the physical and biological functions of extracellular matrix (ECM), which is the natural scaffold for tissue cells [2, 6]. In addition to its fundamental roles in supporting cell growth and guiding tissue formation, one key feature of ECM is its affinity to sequester and stabilize numerous growth factors (GFs) that are key mediators in healing process. These GFs are stabilized by a class of ECM component, glycosaminoglycans (GAGs) [7-9], that protect them from degradation, enrich their local

concentration and enable them to interact with cellular receptors [10, 11]. Thus, establishing proper binding of GFs to a matrix is an essential pre-requisite for GFs to exert their biological actions [12, 13].

Since GFs are known to be key mediators in healing and need stabilization for exerting their therapeutic effect [14], various biomaterials scaffolds have been designed for application as wound dressings that have ability to bind and stabilize a number of GFs exogenously and deliver them at the wound bed upon application. [15-18] Such dressings have made an attempt to recapitulate the sequester and release mechanism of ECM to a certain extent, by immobilizing and stabilizing these exogeneous proteins (sequester) and releasing them at the wound site upon application (release). Though such dressings have been proven effective in accelerating tissue regeneration by utilizing the therapeutic effect of exogenous GFs, they have faced challenges such as delivering adequate amount of these GFs, often delivering them in supraphysiological levels that have serious concerns.[19, 20] Moreover, there have always been concern over the low stability of these exogenous proteins, which often cause a loss in their efficiency even before they are delivered at wound bed [21, 22]. Also, delivering them exogenously results in increasing the treatment cost.

Due to concerns over efficacy and safety of these exogenous proteins, recent researches moved towards fabrication of smart dressings in the form of various hydrogels [23-25], nanofibers [26, 27], sponges [28, 29] and films [30]; which can provide certain cues at the injury site to accelerate the healing process without depending on exogenous GFs. Though these dressings have been proven to be effective in promoting regeneration, they fail to consider the therapeutic effect of GFs, which are principle mediators in tissue

regeneration. Also, neither of these two strategies can hardly enrich endogenous GFs and fail to provide an insight into how the stabilization of endogenous GFs can be effective and indispensable in tissue regeneration.

The key to overcome this challenge lies in fabrication of dressings, that can sequester and stabilize GFs that are released at wound site. At injury site, concentration of GFs increases drastically to initiate angiogenesis and propagate tissue regeneration. These mainly include pro-regenerative GFs, vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF), as reported previously. [31-34] However, due to loss of ECM at injury site, they cannot be sequestered which leaves them unstable and unprotected. Application of a 3-D, GF sequestering matrix will not only provide a physical barrier but can also act as an ECM mimicking GF reservoir. Such dressings can stabilize the released GFs activity and can enhance their therapeutic effect and thereby the process of tissue regeneration. This can go towards fabrication of dressings that can promote tissue regeneration with minimum dependency on exogenous GFs while utilizing the power of endogenous GFs by sequestering and stabilizing them.

In this chapter, the fabricated Hep-PG nanofabrics were employed as wound dressings to evaluate their potential in effective skin tissue regeneration. Endowed with properties of ideal wound dressings and capability in sequestering exogenous and endogenous GFs, Hep-PG nanofabrics promoted scarless and effective tissue regeneration within 14 days of treatment, by utilizing the synergistic effect between exogenously loaded bFGF and sequestered endogenous pro-regenerative GFs. Here we demonstrated a novel biomaterial-based approach with inclusion of heparin, to sequester GFs from exogenous and endogenous source, and how a synergistic effect of exogenous and endogenous GFs

can lead to proper tissue regeneration. This was the first study till date which evaluated a combined role of exogenous and endogenous GFs based on heparin in achieving tissue regeneration.

5.2 Materials and method

5.2.1 In-vivo rat model

6-week-old SD rats (male, SLC, Japan) weighing 180 g were used for in vivo full-thickness skin wound healing studies. All animals were treated and analyzed according to a protocol approved by the Japanese Association for Laboratory Animal Sciences (JALAS). Animals were anesthetized with exposure to isoflurane vapors and then shaved on the back. An 8 mm-diameter biopsy puncher was used to create wounds along the dorsal side of the skin (Figure 5-1a). Two wounds were created on each rat and four rats were used for each time point. Wounds were covered using PG nanofibrous dressings with or without heparin functionalization as shown in figure 5-1b.

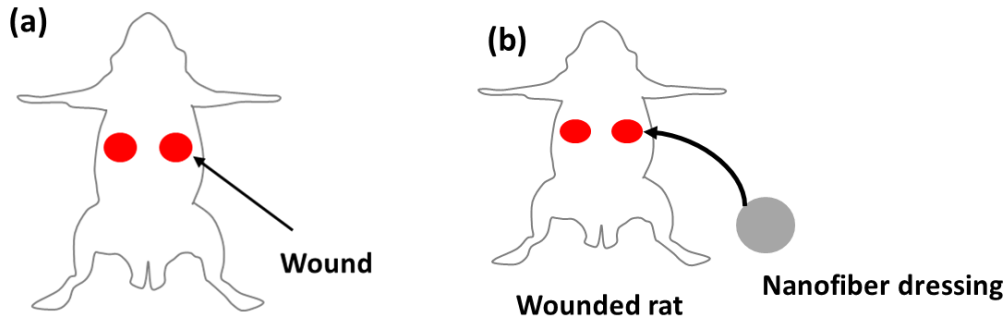


Figure 5-1. (a) Full thickness wound generation at back using biopsy punch; (b) Circular discs of nanofibers were placed on wounds as dressings.

Three conditions of treatment were used. bFGF (+) and bFGF (-) groups represents wounds dressed with PG nanofibrous dressings functionalized with heparin but with or

without pre-loaded bFGF. Control groups represents wound dressed with PG nanofibrous dressings with no heparin functionalization. The details are listed in table below.

Table 5-1: Treatment groups

Treatment group	Effect
bFGF (+)	Hep-PG nanofibers with preloaded bFGF 1. Exogenous bFGF effect 2. Endogenous GFs effect
bFGF (-)	Hep-PG nanofibers but no exogenous GF was loaded • To evaluate endogenous GF effect
Control	PG nanofibers • No GF delivery (neither from exogenous nor from endogenous source)

5.2.2 Evaluation of wound area

Changes in the wound areas were measured using ImageJ software (NIH, Bethesda, MD, USA) at days 0, 7 and 14. At each time point, animals were euthanized by exposure to isoflurane vapor.

5.2.3 HE and MT staining evaluation

At each time point, animals were euthanized by exposure to isoflurane vapor. The surrounding skin and muscle, including the wound areas, were then removed and fixed in 10% neutral, buffered formalin. Tissue samples were embedded in paraffin and sectioned. Haematoxylin and eosin (HE) staining and Masson`s Trichrome (MT) staining was performed to analyze the skin tissue sections.

5.2.4 Immunohistochemical analysis

The sections were also analyzed for immunohistochemistry analysis (IHC) for CD68 to evaluate inflammatory levels. For this the sections were pre-treated in a microwave oven for 20 min at 98°C. Immunohistochemical stain was performed with antibody of anti-CD68 (rabbit polyclonal; ab125212; 1:200; abcam). The number of CD68-positive macrophages was evaluated by total counts at three high-power (X400) fields in the inflammatory lesion.

5.2.5 Statistical analysis

Results are presented as mean ± standard deviation. The difference between the two groups was evaluated using a two-tailed Student's t-test. *$p<0.05$ was considered as significant; **$p<0.01$ was considered highly significant.

5.3 Results and discussion

5.3.1 Wound area evaluation

To evaluate the process of healing, wounds were observed at days 0, 7 and 14 days and the wound area was calculated. Figure 5-2a shows the images of wounds treated using different nanofiber groups (bFGF (+) represents treatment using Hep-PG nanofiber with externally loaded bFGF; bFGF (-) represents treatment using Hep-PG nanofibers but no GF was loaded externally; Control represents treatment with non-heparin conjugated nanofibers). The rats in the bFGF (+) group shows accelerated healing with completely regenerated skin and the absence of scarring at the end of 2 weeks. In contrast, rats in the bFGF (-) group showed slower healing; wounds were not completely healed at the end of 2 weeks of treatment and some amount of scarring can be observed. Healing was the slowest in the control group with large amount of unhealed area post 14 days of treatment.

The wound areas on different days were plotted for analysis (Figure 5-2b). Faster wound closure rate (**$p<0.01$) in bFGF (+) groups compared to bFGF (-) groups confirms better therapeutic effect due to combined effect of externally loaded bFGF and sequestered endogenous GFs. In contrast, control groups showed highest wound area due to absence of both endogenous and exogenous GFs.

From wound closure rates, we were able to support our hypothesis, that sequestering and stabilizing endogenous GFs, can lead to a proper tissue regeneration. This was evident form different in healing levels between bFGF (-) groups and control groups. Further complete regeneration in bFGF (+) groups demonstrates that by supplying a limited amount of GF from exogenous source and endowing biomaterial with endogenous GFs sequestration ability, these exogenous and endogenous proteins can have strong synergistic effect to accelerate the healing process. Such findings can be a valuable contribution in the field of wound care and management and can go towards reduction in treatment cost by utilizing the power of endogenous pro-regenerative GFs.

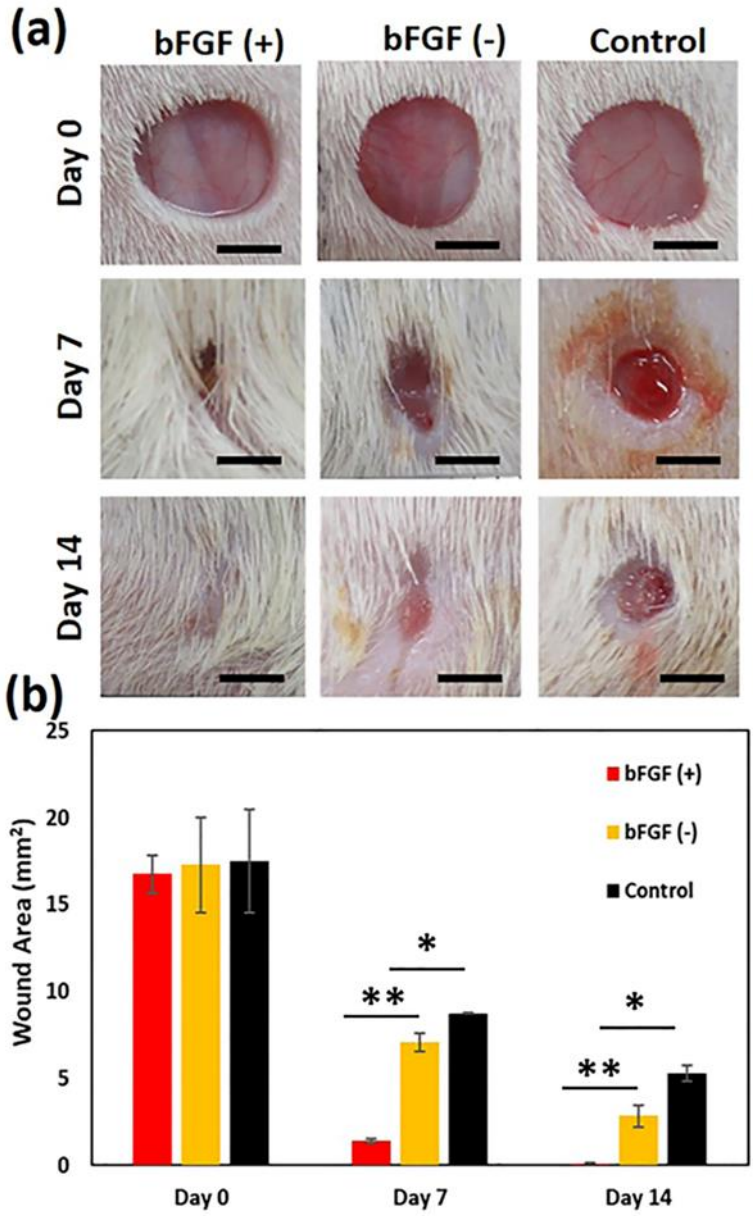


Figure 5-2. Analysis of wound on days 0, 7, and 14. (a) Images showing wounds on respective days. (b) Surface areas of the wounds on the respective days (n=8). Scale bars: 4mm. *$p < 0.05$; **$p < 0.01$.

5.3.2 HE evaluation

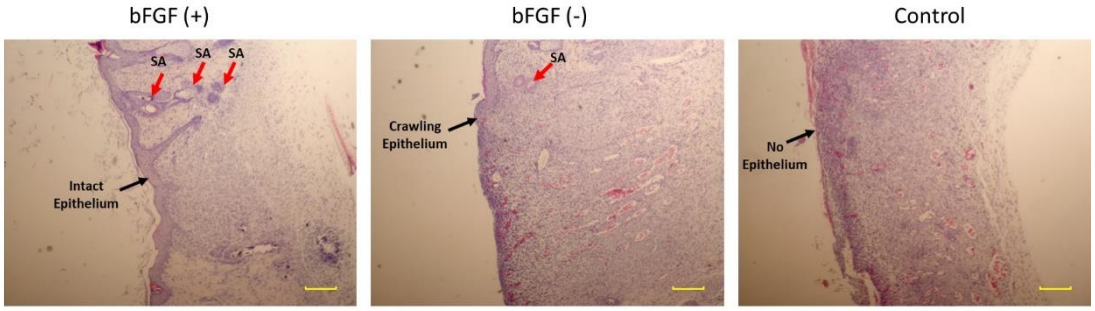
Figure 5-3 shows HE stained sections of the rats under different groups at day 7. As seen from figure 5-3a, rats under bFGF (+) groups showed well defined epidermal and dermal

junctions. Wound contraction was finished at day 7, which was indicated by presence of intact epithelium. Also, lesser inflammatory level was evident from lesser number of protruding blood vessel. Also, skin appendages started to develop. All these parameters were testimony to the ending of inflammatory phase and beginning of late proliferative phase.

In contrast, rats under bFGF (-) groups still lacks presence of well-defined epidermal and dermal junctions. However, crawling epithelium as well as developing skin appendages were testimony to finishing of inflammatory phase and beginning of early proliferative phase.

The condition of rats under control groups at day 7 looked worst. No epidermal-dermal junctions, no epithelium, no skin appendages and excess amount of protruding blood vessels were testimony that wounds were still under inflammatory phase. The afore mentioned parameters have also been shown in figure 5-3b, which showed that healing at day 7 was in late proliferative phase in bFGF (+) group, at early proliferative phase in bFGF (-) group and still in inflammatory phase under control groups.

(a)



(b)

Treatment Group	Wound Contraction	Epithelia Status	Blood vessels	Skin appendages
bFGF (+)	Over	Intact	Little	Start to develop
bFGF (-)	Begin	Crawling	Moderate	Start to develop
Control	Not started	Absent	High	Not yet

Figure 5-3. (a) HE stained tissue sections of rats under different treatments at Day 7. SA indicates skin appendages; (b) Table demonstrating status of various parameter at day 7 from HE staining. *Scale bars: 200 µm.*

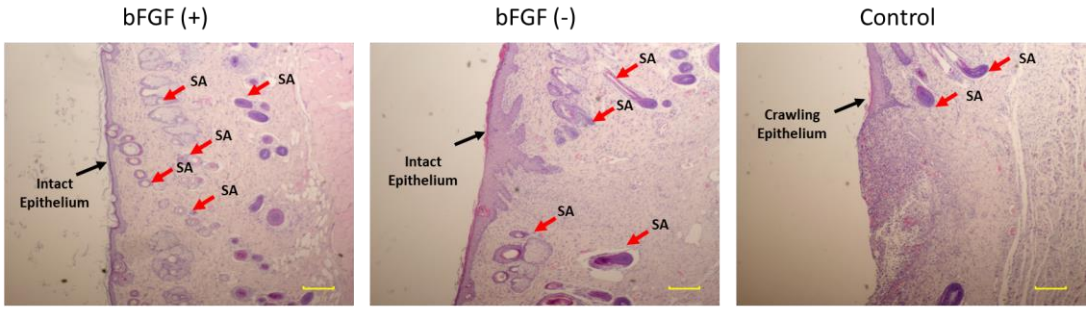
HE stained sections of rats after 14 days of treatment are shown in figure 5-4. As seen from figure 5-4a, well-defined epidermal-dermal junctions were present. Also, negligible blood vessels and high amount of skin appendages were testimony to perfect regeneration under bFGF (+) groups.

In contrast, rats under bFGF (-) groups showed well-defined epidermal-dermal junctions with presence of intact epithelia. Also, many skin appendages can be seen, and wound contraction was over. All these afore parameters were testimony that wounds were in late proliferative phase.

For rats under control groups, crawling epithelium with little mount of developing skin appendages were testimony that inflammatory phase was over and wounds were under initial proliferative phase. These parameters have also been mentioned in figure 5-4b, that

showed perfect regeneration under bFGF (+) group, moderate regeneration under bFGF (-) group and sections looked still healing under control groups.

(a)



(b)

Treatment Group	Wound Contraction	Epithelia Status	Blood vessels	Skin appendages
bFGF (+)	Over	Intact	Little	Developed
bFGF (-)	Over	Intact	Little	Developing
Control	Begin	Crawling	Moderate	Start to develop

Figure 5-4: (a) HE stained tissue sections of rats under different treatments at Day 14. SA indicates skin appendages; (b) Table demonstrating status of various parameter at day 14 from HE staining. *Scale bars: 200 µm.*

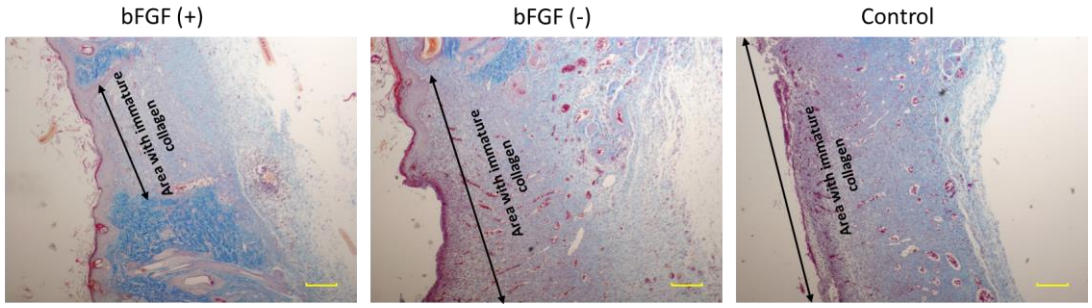
5.3.3 MT evaluation

The results of MT staining at day 7 is shown in figure 5-5. As seen from figure 5-5a, tissue sections of rats under bFGF (+) groups showed only little amount of immature collagen. Most of area was healed with well-defined collagen fibrils. Also, skin appendages can be seen developing with minimum protruding blood vessels.

In contrast, sections of rats under bFGF (-) groups showed larger amount of area with immature collagen. Also, many blood vessels were seen protruding. However, crawling epithelium can be seen indicating wound contraction started.

Control groups showed highest amount of area with immature collagen at day 7. Also, no epithelium was visible, and no skin appendages were visible. The afore mentioned parameters level have been shown in figure 5-5b. Overall, the results at day 7 were in accordance with HE stained sectioned, which indicated late proliferative phase under bFGF (+) sections, early proliferative phase under bFGF (-) sections and wounds were still in inflammatory phase under control groups.

(a)



(b)

Treatment Group	Wound Contraction	Epithelia Status	Blood vessels	Immature collagen area
bFGF (+)	Over	Intact	Little	Less
bFGF (-)	Begin	Crawling	Moderate	Moderate
Control	Not started	Absent	High	High

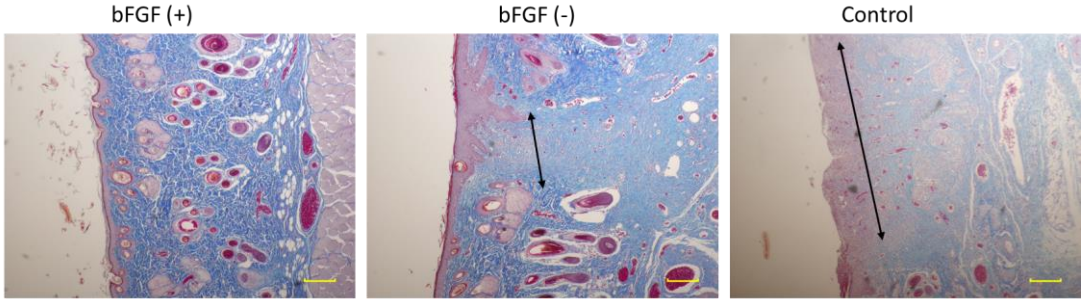
Figure 5-5. (a) MT stained tissue sections of rats under different treatments at Day 7; (b) Table demonstrating status of various parameter at day 14 from MT staining. *Scale bars: 200 µm.*

Day 14 evaluations of MT are shown in figure 5-6. As seen from figure 5-6a, tissue sections of bFGF (+) groups showed well-defined mature collagen fibril with lots of skin appendages that was a testimony to perfect tissue regeneration.

In contrast, rats under bFGF (-) groups showed intact epithelium indicating contraction was finished. Also. Only small amount of area can be seen with immature collagen, indicating that the wounds were in late proliferative phase.

Control group section on the other hand, start to show wound contraction by day 14. Crawling epithelium was visible, however large amount of area with immature collagen can be seen. Also, some skin appendages were visible. All these parameters indicate the wounds were in early proliferative phase, which is marked by wound contraction, collagen deposition and development of skin appendages. The afore mentioned parameters are further demonstrated in figure 5-6b, which clearly indicate perfect regeneration under bFGF (+) group, moderate under bFGF (-) groups and slowest in control groups. The results of HE and MT staining were in accordance with the analysis made from direct wound observation.

(a)



(b)

Treatment Group	Wound Contraction	Epithelia Status	Blood vessels	Immature collagen area
bFGF (+)	Over	Intact	Little	None
bFGF (-)	Over	Intact	Little	Little
Control	Begin	Crawling	Moderate	High

Figure 5-6. (a) MT stained tissue sections of rats under different treatments at Day 14; (b) Table demonstrating status of various parameter at day 14 from MT staining. *Scale bars: 200 µm.*

5.3.4 Immunohistochemical analysis

Tissue sections were also analyzed for inflammatory levels, by performing IHC analysis for CD68 post 14 days of treatment (Figure 5-7). CD68 stains migratory macrophages, which invades the wound bed during the inflammatory phase of healing. Though inflammatory phase is important for healing process, it should gradually decrease with time, as prolonged inflammation leads to scar formation. This is indicated by lower number of macrophages in the wound bed as tissue regenerates [35]. As seen from Figure 5-7, lesser number of macrophages were visible in tissue sections of bFGF (+) groups post 14 days of treatment (indicated by arrow), indicating minimum inflammation. The number looked little higher in bFGF (-) groups, indicating moderate inflammation and highest in tissue sections of control groups, indicating severe inflammation.

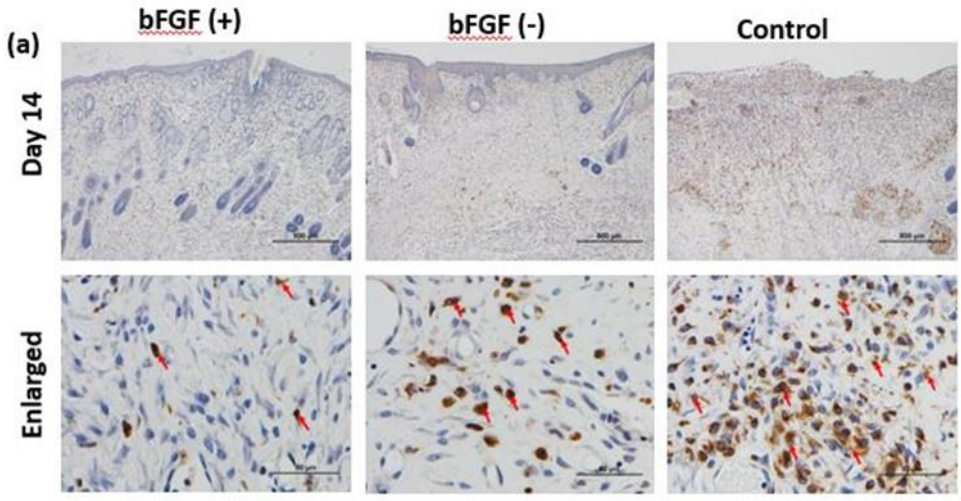


Figure 5-7: Macrophages infiltration at wound sites. (a) Immunohistochemical staining of CD68 in wound site at 14 days after treatment.

5.3.5 Quantification of various parameters: Skin appendages, Scar width, protruding blood vessels and CD68$^+$ cells

The level of healing was also evaluated by quantifying different parameters that are markers of proper healing. Figure 5-8 shows quantified parameters. Proper healing is indicated by the formation of intact epithelia, well-defined epidermis-dermis junctions, the presence of mature collagen fibrils, the formation of skin appendages, minimum scar formation and a reduction in blood vessels as the tissue becomes mature [36-38]. Also, proper healing is marked by decrease in number of CD68$^+$ cells in wound bed. All these afore parameters were quantified and shown in figure 8. The healing process was almost complete within two weeks of treatment in bFGF (+) treated groups. This was indicated by the significantly higher number of skin appendages which is a sign of proper healing (Figure 5-8a). There were also significantly fewer blood vessels, which indicated lower inflammation in the newly born granulation, compared to rats in the bFGF (-) group (Figure 5-8b). Minimum scarring was also observed in the bFGF (+) groups (Figure 5-8c). Also, minimum number of CD68$^+$ cells were observed indicating minimum inflammation which is another marker of proper healing (Figure 5-8d).

Although the healing of the wounds treated with heparin-loaded nanofibers without bFGF (bFGF (-) group) was inferior compared to that in the bFGF (+) group, their tissue sections shows well-defined epithelia with the presence of dermis and epidermis junctions. Also, compared to the control group, there were significantly more newly formed skin appendages and fewer blood vessels. Collagen fibril in maturing phase were also present. Although some scarring was observed, the widths of the scars were significantly smaller than that present in the control groups. Also, inflammation levels were moderate as compared to control groups (Figure 5-8).

The control groups have shown the least amount of healing. No well-defined epithelia were observed after fourteen days of treatment. In addition, no dermal and epidermal junctions were observed. A larger number of blood vessels with almost no skin appendages were also observed. This indicated that the wounds were still healing. The skin sections also show a large amount of scarring, which confirms that the healing in control rats was imperfect. Also, severe inflammation levels were observed (Figure 5-8).

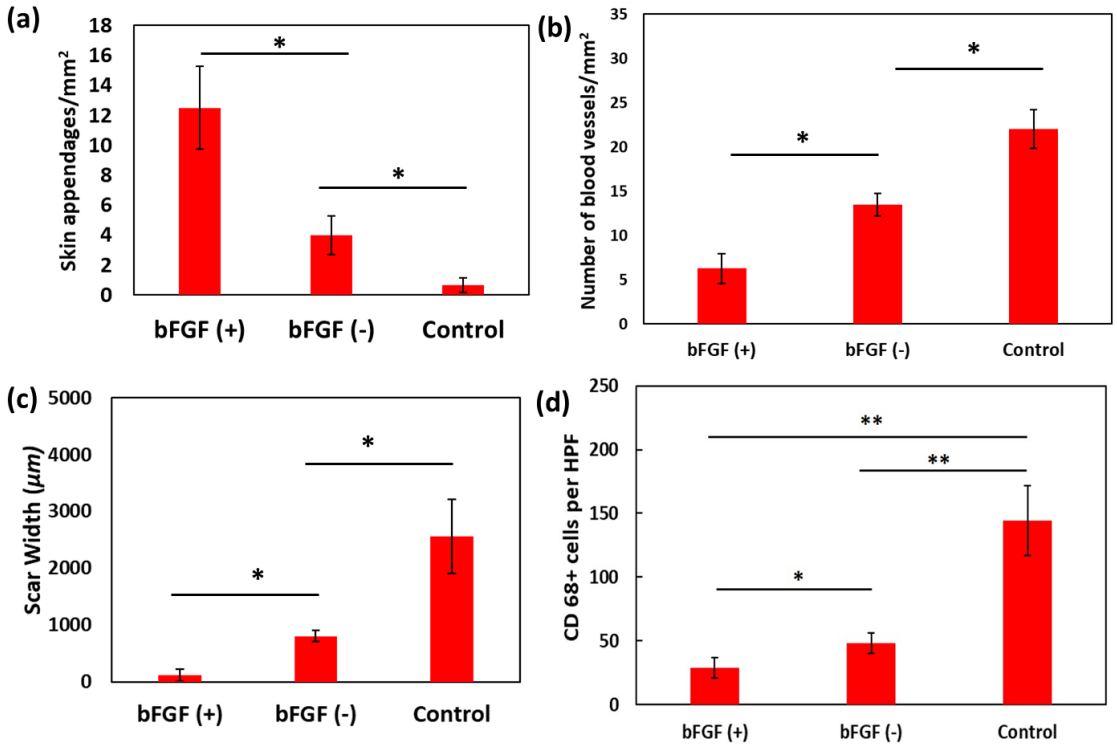


Figure 5-8: Quantification of various parameters that are indicators of healing. (A) Skin appendages; (b) Number of protruding blood vessels; (c) Scar width and (d) $CD68^{+}$ cells/HPF.

Overall, the results of histological evaluations and IHC analysis showed that healing was best under synergistically mediated treatment, moderate under endogenously mediated

treatment and worst when no GFs were employed. The regeneration levels are further summarized in Table 5-1 by indicating levels of parameters from histopathological and IHC analysis, that concludes the level of healing under each treatment groups. As stated previously, proper tissue regeneration is marked by lower inflammatory levels, lower scarring levels, lesser number of macrophages infiltration and formation of epidermal coating. The table further demonstrates excellent healing under bFGF (+) groups, moderate under bFGF (-) groups and worst under control groups.

Table 5-2: Summary of various parameters obtained from histological and IHC analysis

Treatment Group	Inflammatory level	Scarring Level	Macrophage Infiltration	Epidermis Coating
bFGF (+)	Little	Little	Little	Present
bFGF (-)	Moderate	Moderate	Moderate	Present
Control	Altitudes	High	High	Absent

5.4 Discussion

Wound dressings that can stimulate proper tissue regeneration are highly demanded. Over the years, numerous researches have been conducted to fabricate ideal dressings that can promote effective tissue regeneration. Nevertheless, few current biomaterials scaffolds could recapitulate the prominent feature of ECM, to sequester and release GFs which are key meditators in tissue regeneration. It emerges as a pressing need to device a scaffold that can mimic ECM in sequestering the endogenous, pro-regenerative GFs around the site of injury and thereby create a suitable niche to facilitate endogenously driven tissue regeneration.

In this chapter, Hep-PG nanofibers loaded with exogenous bFGF and having endogenous GFs sequestration ability were employed as wound dressings to evaluate their ability in promoting accelerated tissue regeneration using in-vivo full thickness rat wound model. By observing the morphology of wounds and through histological examinations, we showed that our nanofiber matrices not only accelerated the healing process, but also ensured scarless tissue regeneration. The synergistic effect between externally loaded bFGF and sequestered endogenous GFs worked well, and we could obtain an accelerated tissue regeneration. To best of our knowledge, this is first report on the combined effect of endogenous and exogenous GFs in promoting proper tissue regeneration. We believe such valuable findings can find its application towards fabrication of ideal wound dressings.

5.5 Conclusion

This chapter demonstrated an ideal way to fabricate wound dressings with minimum dependency on exogenous GFs and utilizing endogenous GFs therapeutic effect for obtaining cost-effective and accelerated tissue regeneration. Heparin functionalized PCL/gelatin co-spun nanofibers were analyzed for their exogenous and endogenous GF sequestering capability. When employed as wound dressings, a synergistic effect between exogenous bFGF and sequestered endogenous GFs promoted proper tissue regeneration. Taken together, this study demonstrated an innovative and feasible biomaterials-based approach to exploit the power of endogenous GFs in promoting accelerated tissue regeneration with minimum dependency on exogenous GFs.

References

[1]. Singer, A. J.; Clark, R. A., Cutaneous wound healing, N. Engl. J. Med. 341(10) (1999) 738-746.

[2]. Gurtner, G. C.; Werner, S.; Brandon, Y.; Longaker, M. T., Wound repair and regeneration, Nature 453(7193) (2008) 314.

[3]. Martin, P., Wound healing--aiming for perfect skin regeneration. Science 276(5309) (1997) 75-81.

[4]. Branski, L. K.; Gauglitz, G. G.;Herndon, D. N.; Jeschke, M. G., A review of gene and stem cell therapy in cutaneous wound healing, Burns 35(2) (2009) 171-180.

[5]. Boateng, J. S.; Matthews, K. H.; Stevens, H. N.; Eccleston, G. M., Wound healing dressings and drug delivery systems: a review, J. Pharm. Sci. 97(8) (2008) 2892-2923.

[6]. Forbes, S. J.; Rosenthal, N., Preparing the ground for tissue regeneration: from mechanism to therapy, Nat. Med. 20(8) (2014) 857-869.

[7]. Freudenberg, U.; Liang, Y.; Kiick, K. L.; Werner, C., Glycosaminoglycan-Based Biohybrid Hydrogels: A Sweet and Smart Choice for Multifunctional Biomaterials, Adv. Mater. 28(40) (2016) 8861-8891.

[8]. Martino, M. M.; Brkic, S.; Bovo, E.; Burger, M.; Schaefer, D. J.; Wolff, T.; Gürke, L.; Briquez, P. S.; Larsson, H. M.; Gianni-Barrera, R.; Hubbell, J. A.; Banfi, A., Extracellular Matrix and Growth Factor Engineering for Controlled Angiogenesis in Regenerative Medicine, Front. Bioeng. Biotechnol. 3(45) (2015).

[9]. Briquez, P. S.; Clegg, L. E.; Martino, M. M.; Gabhann, F. M.; Hubbell, J. A., Design principles for therapeutic angiogenic materials, Nat. Rev. Mater. 1(1) (2016) 15006.

[10]. Wang, C.; Poon, S.; Murali, S.; Koo, C.-Y.; Bell, T. J.; Hinkley, S. F.; Yeong, H.; Bhakoo, K.; Nurcombe, V.; Cool, S. M., Engineering a vascular endothelial growth factor 165-binding heparan sulfate for vascular therapy, Biomaterials 35(25) (2014) 6776-6786.

[11]. Lee, K.; Silva, E. A.; Mooney, D. J., Growth factor delivery-based tissue engineering: general approaches and a review of recent developments, J. R. Soc. Interface 8(55) (2011) 153-170.

[12]. Hudalla, G. A.; Murphy, W. L., Biomaterials that Regulate Growth Factor Activity via Bioinspired Interactions, Adv. Funct. Mater. 21(10) (2011) 1754-1768.

[13]. Belair, D. G.; Murphy, W. L., Specific VEGF sequestering to biomaterials: Influence of serum stability, Acta Biomater. 9 (11) (2013) 8823-8831.

[14]. Grazul-Bilska, A. T.; Johnson, M. L.; Bilski, J. J.; Redmer, D. A.; Reynolds, L. P.; Abdullah, A.; Abdullah, K. M., Wound healing: the role of growth factors, Drugs Today (Barc) 39(10) (2003) 787-800.

[15]. Wang, Z.; Wang, Z.; Lu, W. W.; Zhen, W.; Yang, D.; Peng, S., Novel biomaterial strategies for controlled growth factor delivery for biomedical applications, NPG Asia Mater. 9(10) (2017) e435-e435.

[16]. Lee, M. S.; Ahmad, T.; Lee, J.; Awada, H. K.; Wang, Y.; Kim, K.; Shin, H.; Yang, H. S., Dual delivery of growth factors with coacervate-coated poly (lactic-co-glycolic acid) nanofiber improves neovascularization in a mouse skin flap model, Biomaterials 124 (2017) 65-77.

[17]. Whelan, D.; Caplice, N.; Clover, A., Fibrin as a delivery system in wound healing tissue engineering applications, J. Control. Release 196 (2014) 1-8.

[18]. Niu, Y.; Li, Q.; Ding, Y.; Dong, L.; Wang, C., Engineered delivery strategies for enhanced control of growth factor activities in wound healing, Adv. Drug Deliv. Rev. 146 (2019) 190-208.

[19]. Liu, Z.; Yan, R.; Al-Salman, A.; Shen, Y.; Bu, Y.; Ma, J.; Luo, D.-X.; Huang, C.; Jiang, Y.; Wilber, A., Epidermal growth factor induces tumour marker AKR1B10 expression through activator protein-1 signalling in hepatocellular carcinoma cells, Biochem. J. 442(2) (2012) 273-282.

[20]. Mitragotri, S.; Burke, P. A.; Langer, R., Overcoming the challenges in administering biopharmaceuticals: formulation and delivery strategies, Nat. Rev. Drug Discov. 13(9) (2014) 655-672.

[21]. Schultz, G. S.; Sibbald, R. G.; Falanga, V.; Ayello, E. A.; Dowsett, C.; Harding, K.; Romanelli, M.; Stacey, M. C.; Teot, L.; Vanscheidt, W., Wound bed preparation: a systematic approach to wound management, Wound Repair Regen. 11 (2003) S1-S28.

[22]. Jain, R.; Agarwal, A.; Kierski, P. R.; Schurr, M. J.; Murphy, C. J.; McAnulty, J. F.; Abbott, N. L., The use of native chemical functional groups presented by wound beds for the covalent attachment of polymeric microcarriers of bioactive factors, Biomaterials 34(2) (2013) 340-352.

[23]. Zhang, X.; Zhang, G.; Zhang, H.; Liu, X.; Shi, J.; Shi, H.; Yao, X.; Chu, P. K.; Zhang, X., A bifunctional hydrogel incorporated with CuS@ MoS2 microspheres for disinfection and improved wound healing, Chem. Eng. J. 382 (2020) 122849.

[24]. Zhou, Q.; Wang, J.; Liang, Y.; Yang, H.; Li, Q.; Li, Q.; Liao, D.; Liu, Y.; Liu, H. B., Development of an Intelligent Photosensitive Antibacterial Wound Dressing: Simultaneous Detection and Treatment of Bacterial Infection for Accelerated Wound Healing, ChemNanoMat 6(4) (2020) 516-523.

[25]. Thi, P. L.; Lee, Y.; Tran, D. L.; Thi, T. T. H.; Kang, J. I.; Park, K. M.; Park, K. D., In situ forming and reactive oxygen species-scavenging gelatin hydrogels for enhancing wound healing efficacy, Acta Biomater. 103 (2020) 142-152.

[26]. Wu, K.; Wu, X.; Chen, M.; Wu, H.; Jiao, Y.; Zhou, C., H2O2-responsive Smart Dressing for visible H2O2 Monitoring and accelerating wound healing, Chem. Eng. J. 387 (2020) 124127.

[27]. He, J.; Liang, Y.; Shi, M.; Guo, B., Anti-oxidant electroactive and antibacterial nanofibrous wound dressings based on poly (ε-caprolactone)/quaternized chitosan-graft-polyaniline for full-thickness skin wound healing, Chem. Eng. J. 385 (2020) 123464.

[28]. Fang, Y.; Xu, Y.; Wang, Z.; Zhou, W.; Yan, L.; Fan, X.; Liu, H., 3D porous chitin sponge with high absorbency, rapid shape recovery, and excellent antibacterial activities for noncompressible wound, Chem. Eng. J. 388 (2020) 124169.

[29]. Zhang, K.; Bai, X.; Yuan, Z.; Cao, X.; Jiao, X.; Li, Y.; Qin, Y.; Wen, Y.; Zhang, X., Layered nanofiber sponge with an improved capacity for promoting blood coagulation and wound healing, Biomaterials 204 (2019) 70-79.

[30]. Tang, Q.; Lim, T.; Wei, X.-J.; Wang, Q.-Y.; Xu, J.-C.; Shen, L.-Y.; Zhu, Z.-Z.; Zhang, C.-Q., A free-standing multilayer film as a novel delivery carrier of platelet lysates for potential wound-dressing applications, Biomaterials 255 (2020) 120138.

[31]. Ferrara, N.; Gerber, H.-P.; LeCouter, J., The biology of VEGF and its receptors, Nat. Med. 9(6) (2003) 669-676.

[32]. Miraliakbari, R.; Francalancia, N. A.; Lust, R. M.; Gerardo, J. A.; Ng, P. C.; Sun, Y. S.; Chitwood Jr, W. R., Differences in myocardial and peripheral VEGF and KDR levels after acute ischemia, Ann. Thorac. Surg. 69(6) (2000) 1750-1754.

[33]. Golebiewska, E. M.; Poole, A. W., Platelet secretion: From haemostasis to wound healing and beyond, Blood Rev. 29 (3) (2015) 153-162.

[34]. DiPietro, L. A., Wound healing: the role of the macrophage and other immune cells, Shock 4(4) (1995) 233-240.

[35]. Qi, C.; Xu, L.; Deng, Y.; Wang, G.; Wang, Z.; Wang, L., Sericin hydrogels promote skin wound healing with effective regeneration of hair follicles and sebaceous glands after complete loss of epidermis and dermis, Biomater. Sci. 6(11) (2018) 2859-2870.

[36]. Chantre, C. O.; Campbell, P. H.; Golecki, H. M.; Buganza, A. T.; Capulli, A. K.; Deravi, L. F.; Dauth, S.; Sheehy, S. P.; Paten, J. A.; Gledhill, K., Production-scale fibronectin nanofibers promote wound closure and tissue repair in a dermal mouse model, Biomaterials 166 (2018) 96-108.

[37]. Xie, Z.; Paras, C. B.; Weng, H.; Punnakitikashem, P.; Su, L.-C.; Vu, K.; Tang, L.; Yang, J.; Nguyen, K. T., Dual growth factor releasing multi-functional nanofibers for wound healing, Acta Biomater. 9(12) (2013) 9351-9359.

[38]. Wu, Z.; Hong, Y., Combination of the Silver–Ethylene Interaction and 3D Printing to Develop Antibacterial Superporous Hydrogels for Wound Management, ACS Appl. Mater. Interfaces 11(37) (2019) 33734-33747.

Chapter 6:

General Conclusion and Future Study

6.1 Conclusion

Ideal wound dressings that can promote proper tissue regeneration our highly demanding. As discussed previously, since growth factors (GFs) are actively involved in tissue regeneration, their delivery is indispensable in proper tissue regeneration. However, since exogenous delivery have issue related to safety and concerns, the current research made an attempt to provide a solution through stabilizing endogenous pro-regenerative factors through inclusion of ECM component into a nanofibrous dressing. Firstly, fabrication of dressing was done using co-axial electrospinning technique and optimization was made to fabricate Gelation coated PCL nanofibrous dressings that can balance between mechanical characteristics and biocompatibility. The fabricated dressings were extensively characterized in-vitro for their suitability as wound dressings. At next stage, to endow GFs sequestration ability into fabricated nanofabrics, heparin conjugation was achieved via EDC/NHS coupling. These heparin functionalized PCL/gelatin co-spun nanfabrics, called as Hep-PG were analysed for their ability to load exogenous GFs and for their ability to sequester and stabilize endogenous pro-regenerative GFs released at regenerative site. Finally, these Hep-PG nanofibers with capability to sequester both exogenous and endogenous GFs were employed as wound dressing to promote synergistically mediated tissue regeneration by utilizing combined effect of externally loaded bFGF and sequestered pro-regenerative GFs. We were able to defend our hypothesis and these Hep-PG nanofibers were able to promote complete tissue regeneration within 14 days of treatment. Taken together, this study demonstrated an

innovative and feasible biomaterials-based approach to exploit the power of endogenous GFs in promoting accelerated tissue regeneration with minimum dependency on exogenous GFs.

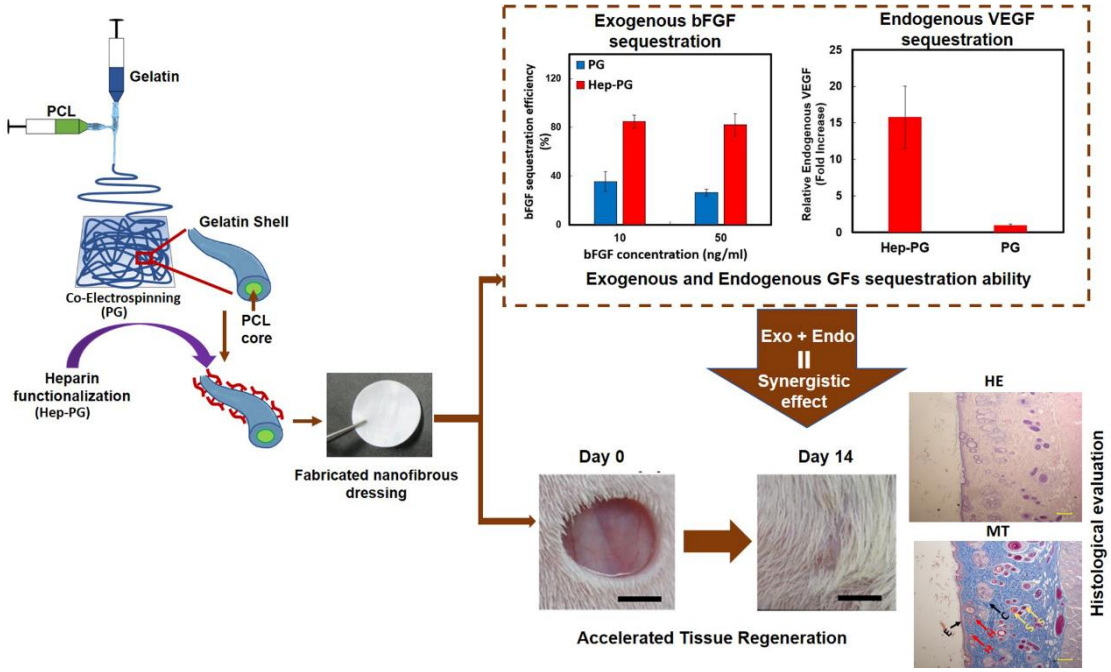


Figure 6-1: Summary of research shown pictorially

6.2 Future Study

In the current study, we demonstrated the potential of Hep-PG nanofibers as a potent for sequestering both exogenous and endogenous GFs and in mediating complete tissue regeneration by utilizing the power of these proteins. This thesis provides an ideal biomaterial-based approach to utilize endogenous GFs as a source while reducing the supply of exogenous delivery.

However, we showed a synergistic effect of externally loaded bFGF and endogenous GFs in this process. The aim of my research was to minimize dependency on exogenous factors, however still some amount was utilized. Exogenous delivery worked well with

endogenous sequestration ability, however in future work I want to totally ignore dependency on exogenous factors and just utilize properties of my dressing for obtaining effective regeneration.

For this, my target is to employ the same co-electrospinning technique, but to replace gelatin with quaternized chitosan-graft- polyaniline (QCSP) as shell component. QCSP has been well reported to promote tissue regeneration due to its elector-activity and antioxidant properties. By functionalizing such core-shell type PCL/QCSP nanofabrics with heparin, further these dressings can be incorporated with ability in sequestering endogenous pro-regenerative GFs. Having antioxidant, electroactive properties as well as anti-bacterial characteristics coupled with endogenous GFs sequestration abilities, such dressings can utilize the synergistic effects of all afore mentioned parameters and can promote accelerated tissue regeneration. Thus, a dressing with no dependency on exogenous factors can be developed. This is my current study and it’s still ongoing.

Acknowledgement

Firstly, I am very grateful to my supervisor, ***Prof. Hiroyuki Ijima***, for his patience and valuable guidance during the period of my Ph.D. study. He taught me how to study and think, what to innovate and dedicate. Under his guidance, I gained not only a wealth of knowledge but also advanced concepts.

I am also thankful to ***Prof. Masamichi Kamihira and Prof. Noriho Kamiya***, for their critical views on modifying this thesis.

I am thankful to ***Assistant Prof. Yusuke Sakai***, who always helped me during discussions and during operating various equipment in lab. Also, his kind and friendly attitude always helped me to stay motivated.

I am also thankful to ***Prof. Matsune and Prof. Mizumoto*** for providing me valuable guidance. Special thanks to ***Prof. Matsune*** for helping me during TEM operations.

I would also like to acknowledge ***Prof. Aishima*** for helping me in the studies related to immunohistochemical analysis.

I should also thank ***JICA*** for their financial and moral support during my PhD. I would like to especially thank all JICA coordinators who were always faithful and showed trust in me that motivated me to continue my study at Kyushu University. Special thanks to ***Sakono san*** and ***Masamoto san*** for boosting my morale at times when I felt low.

Apart from this, I would also like to thank all my laboratory friends at Kyushu University for their caring and helping nature during my research period. I specially thank ***Mr. Xu Zhe and Dr. Jannatul Fardous*** for their frequent availability as and when I needed their help and support. A special thanks to all the Indian collaborators, especially to ***Mr. Akshay Joshi***, ***Dr. Tejinder Kaur*** and ***Dr. Senthil Guru***, IIT Delhi, India, for their valuable suggestions and contributions. Finally, I would like to thank my family, specially my mother, friends in India for their moral and emotional support.