# DEVELOPMENT OF PVA-NATURAL BIOPOLYMER HYDROGEL INCORPORATED WITH STINGLESS BEE HONEY FOR WOUND HEALING

BY

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# ABSTRACT

When the skin is injured, it would compromise the immune system as well as body's homeostasis. Wound that fail to progress into the normal stage of healing is being recognized as delayed acute wound and chronic wound that could worsen the body condition. Honey is a well establish treatment for wound healing due to its antimicrobial, antioxidants, anti-inflammatory and high sugar contents. First of all, the stingless bee honey was evaluated via *in vitro* in term of antimicrobial and anti-inflammatory properties. 40% w/v concentration of honey displayed a good antimicrobial property against *Staphylococcus aureus* colonies since the honey able to inhibit their growth. In addition, the honey did not reduce the proinflammatory cytokines produced by LPS-activated macrophages. It is due to the immunomodulatory properties of honey that react towards the cytokines depending on the microenvironment of the wound. From the *in vitro* evaluation, 40% w/v concentration of honey was selected to be incorporated with hydrogel. Then, stingless bee-based hydrogel formulation was developed and characterized. Response surface model (RSM) which is consist of factorial design and central composite design was used to develop and optimized the hydrogel. Then, the characterization of the formulation was studied in term of hydrophilicity, polymerization, water vapor transmission rate (WVTR), rheological, drug release, microbial limit test, antimicrobial, stability and interaction between honey and the excipient by using Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR). Formulation 3 displayed a good reaction towards the selected response. Formulation 3 hydrogel has a good swelling ability which could provide a good moisturizing condition to the wound. Formulation 3 exhibited a good WVTR where the value is lower than control formulation. A higher WVTR cause the wound to dry rapidly. In rheological properties, Formulation 3 displayed a clear LVR line while in ATR-FTIR test, there was no substantial interaction observed in comparison with blank hydrogel. In addition, the release profile in Formulation 3 followed diffusion-controlled mechanism where the delivery of the honey is consistent throughout the time. Then, in term of microbial activity, Formulation 3 exhibited a good antimicrobial property and free from pathogenic microorganism. Finally, from the accelerated stability data, Formulation 3 was stable throughout storage period. For *in vivo* study, the blank and honey hydrogel were safe to use on the skin of the rabbit. The healing in honey hydrogel treated group was significantly ($p<0.05$) faster than no treatment group as shown in wound closure percentage and histological assessment. In honey hydrogel treated group, the amount of collagen deposited is abundant and the structure of fibroblast is well organized, this indicated at the end of the animal study, the wound has entered the remodelling phase compared with the no treatment group that still in proliferation phase. Based on all of these results, there are a lot of promising properties of stingless bee honey-based hydrogel such as good hydrophilicity, elasticity, microbial safety, reliable release profile as well as efficacy in wound healing treatment. Hence, it can be concluded that stingless bee honey-based hydrogel has a high potential to be a good wound dressing.

# خلاصة البحث

عندما يُجرح الجلد فإن من الممكن أن يعرض الجهاز المناعي للخطر ويؤثر كذلك على توازن الجسم. تصنف الجروح التي تفشل في التقدم إلى المرحلة الطبيعية للشفاء كجروح حادة وجروح مزمنة متأخرة والتي قد تؤدي إلى تفاقم حالة الجسم. العسل علاج جيد للجروح بسبب احتوائه على مضادات للميكروبات ومضادات للأكسدة ومضادات للالتهاب ولاحتوائه على نسبة عالية من السكر. في البداية تم تقييم عسل النحل الضئيل الإبر (stingless bee) خارج الجسم الحي بناء على الخصائص المضادة للميكروبات والمضادة للالتهابات. أظهر العسل بكثافة 40٪ من الوزن إلى الكتلة خواصا جيدة مضادة لمستعمرات الميكروبات من نوع المكورات العنقودية الذهبية، والزائفة الزنجارية. لم يقلل العسل من السيتوكينات المسببة للالتهابات التي تنتجها البلاعم المفعلة بالـ LPS، ويرجع ذلك إلى الخواص المناعية للعسل التي تتفاعل مع السيتوكينات والتي تعتمد على البيئة الدقيقة داخل الجرح. اعتمادا على نتائج التقييم خارج الجسم الحي تم اختيار العسل بكثافة 40٪ من الوزن إلى الكتلة ليتم دمجه مع هيدروجيل. بعد ذلك تم تطوير وتوصيف تركيبة هيدروجيل المحتوية على النحل. تم استخدام النموذج السطحي الاستجابي (RSM) المتكون من التصميم العاملي والتصميم التركيبي المركزي لتطوير وتحسين الهيدروجيل. بعد ذلك تمت دراسة توصيف التركيبة من حيث الألوفة المائية، والبلمرة، ومعدل انتقال بخار الماء (WVTR)، والريولوجية، وإطلاق العقار، واختبار الحد الميكروبي، الخواص المضادة للميكروبات، والاستقرار التخزيني، والتفاعل بين العسل والسواغ باستخدام طيف الأشعة (ATR-FTIR). أظهرت الصيغة رقم 3 رد فعل جيد تجاه الاستجابة المحددة. وكان لدى الصيغة رقم 3 للهيدروجيل خاصية جيدة للألوفة المائي والتي بإمكانها توفير حالة ترطيب جيدة للجروح. أظهرت الصيغة رقم 3 قيمة WVTR جيدة حيث كانت القيمة أقل من التركيبة الضابطة، حيث تؤدي القيم العالية للـ WVTR إلى الجفاف السريع للجروح. أما بالنسبة للخواص الريولوجية فقد أظهرت الصيغة رقم 3 خطا واضحا للـ LVR، بينما في اختبار ATR-FTIR لم يكن هناك تفاعل كبير مقارنة مع الهيدروجيل الفارغ. اتبع سلوك الإطلاق في الصيغة رقم 3 آلية الانتشار المنظم حيث تم إطلاق العسل باستمرار طوال الوقت. أما من حيث النشاط الميكروبي فقد أظهرت الصيغة رقم 3 خاصية جيدة مضادة للميكروبات وكانت خالية من الميكروبات المسببة للأمراض. وفي النهاية، بناء على بيانات الاستقرار المتسارع، كانت الصيغة رقم 3 مستقرة طوال فترة التخزين. في الدراسة التي تمت داخل الجسم الحي، لم يسبب الهيدروجيل الفارغ والهيدروجيل المحمل بالعسل تهيجا على جلود الأرانب. كان الشفاء في المجموعة المعالجة بالهيدروجيل المحمل بالعسل أسرع بكثير من أي مجموعة علاجيةكما هو واضح في نسبة إغلاق الجروح والتقييم النسيجي. في المجموعة المعالجة بالهيدروجيل المحمل بالعسل، كانت كمية الكولاجين المودعة وفيرة، وكان تنظيم بنية الخلايا الليفية جيدا. وهذا يدل أنه في نهاية الدراسة على الحيوانات قد دخل الجرح في مرحلة إعادة التشكيل مقارنة بالمجموعة الغير المعالجة التي لا زالت في مرحلة التجدد الخلوي. بناءً على كل هذه النتائج، فإنه هناك الكثير من الخصائص الواعدة للهيدروجيل المحمل بعسل النحل الضئيل الإبر مثل الألوفة المائية، والمرونة ، والسلامة الميكروبية، وسلوك الإطلاق الموثوق، بالإضافة
إلى فعاليته في علاج التئام الجروح، والتي قد تكون كافية لتكون هذه التركيبة بمثابة ضمادة جيدة للجروح.

# APPROVAL PAGE

The thesis of Mohd Azri bin Abd Jalil has been approved by the following:

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# DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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# LIST OF ABBREVIATONS

| | |
|---|---|
| ATR-FTIR | Attenuated Total Reflectance-Fourier Transform Infrared |
| CCD | composite design |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | DMSO |
| DOE | Doe Design of Experiment |
| FGF | fibroblast growth factor |
| F-T cycle | Freeze Thaw Cycle |
| IL-6 | interleukin-6 |
| LPS | LPS lipopolysaccharide |
| LVR | linear viscoelastic region |
| MHA | Mullen Hilton agar |
| MHB | Mullen Hilton broth |
| mrsa | Methicillin-resistant *Staphylococcus aureus* MRSA |
| MTT | MTT 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide |
| NPWT | Negative pressure wound therapy |
| PBS | PBS Phosphate Buffer Saline |
| PDGF | platelet-derived growth factor |
| PEG | polyethylene glycol |
| PEO | polyethylene oxide |
| $PGE_2$ | prostaglandin $E_2$ |
| PHEMA | poly(2-hydroxylethyl methacrylate) |
| PNVP | polyvinylpyrrolidone |
| PVA | polyvinyl alcohol |
| ROI | Reactive oxygen intermediates |
| RSM | Response surface modeling |
| TGF- β | transforming growth factor β |
| TNF-α | tumour necrosis factor-α |
| VEGF | vascular endothelial growth factor |
| vWF | von Willebrand factor |
| WVTR | water vapour transmission rate |

# LIST OF SYMBOLS

| | |
|---|---|
| % | Percent |
| < | Less |
| > | More |
| | |
| °C | Degree Celsius |
| µL | Microgram |
| $cm^2$ | Centimeter square |
| g | Gram |
| G’ | Storage modulus |
| G″ | Loss modulus |
| mg | Milligram |
| mL | Millilitre |
| nm | Nanometer |
| Pa | Pascal |
| $R^2$ | Coefficient of determination |
| rpm | Revolution per minute |
| v/v | Volume per volume |
| w/v | Weight per volume |
| τ | Shear stress |

# CHAPTER ONE

# INTRODUCTION

## 1.1 BACKGROUND OF THE STUDY

Wound healing remains one of the major concerns among healthcare practitioners and scientists until this present study. Poor wound healing does not only cause trauma to the patients, but the process itself becomes time-consuming and could cause an enormous drain to the healthcare resources. For example, in the United Kingdom, National Health Services estimated the cost for managing wound cases was approximately £5.3 billion per annum, representing 4% of the total expenditure funded by the government (Guest, Vowden, & Vowden, 2017). In the United States of America, the estimated cost of wound care was nearly $50 billion per annum that covered 5% from the total expenditure spent on Medicare and Medicaid in the USA (Vowden & Vowden, 2016). This number may arise in the future since the prevalence of wounds is increasing due to the ageing population, in addition with other comorbidities factors that could hinder the healing processes such as cardiac disease, diabetes, and obesity (Gould et al., 2015). Therefore, a careful wound healing management should be considered seriously to reduce the burden and improve the outcome of the wound healing cases.

Over the years, there are a lot of methods and inventions introduced to overcome wound healing problems. Since the beginning of the 20$^{th}$ century, advanced technologies in polymerisation research has contributed refreshing materials for wound healing products. Several polymers were introduced as wound dressings' materials such as nylon, polyurethane, polyester, acrylic, and rayon (Martin et al.,

2013). On the other hand, cellulose, dextran, alginate, chitin, and chitosan were chosen as ingredients for the natural-friendly products (Mogoşanu & Grumezescu, 2014). A few studies indicated that moist environment is the ideal condition to improve wound healing process (Goh, Hwang, & Tae, 2016; Kurhade, Momin, Khanekar, & Mhatre, 2013; Pinho, Grootveld, Soares, & Henriques, 2014). Therefore, a dressing that can imbibe sufficient water content was developed and it is now known as hydrogel.

The hydrogel has a promising attribute in drug delivery systems due to their high-water content that can imitate similar environment as the human body tissue and provides good biocompatibility to encapsulate hydrophilic drugs (Li & Mooney, 2016). Also similar to other dressings, the hydrogel consists of synthetic polymers ingredients such as polyvinyl alcohol (PVA), polyvinylpyrrolidone (PNVP), poly(2-hydroxylethyl methacrylate) or PHEMA, polyethylene oxide (PEO), and polyethylene glycol (PEG) (González-Torres et al., 2018; Haryanto, Singh, Huh, & Kim, 2016; Passos et al., 2016). Meanwhile, the natural polymers used to synthesis the hydrogels were alginate, chitin derivatives, alginic acid, starch, dextran, hyaluronic acid, and pectin (Alhaique et al., 2016; Kamoun, Kenawy, & Chen, 2017; Kowalski, Kijowska, Witczak, Kuterasiński, & Lukasiewicz, 2019; Yuan et al., 2017). Both types of ingredients have their own benefits and weaknesses. Synthetic polymers contain strong chemical structure due to the hydrolysable moieties that can delay the degradation rate while natural polymers are more biocompatible to the human body since they can be naturally degraded by human enzymes which indirectly yield biocompatible by-products (Ahmadi, Oveisi, Samani, & Amoozgar, 2015).

The use of natural products that are incorporated with synthetic polymers has been a subject of interest since both materials have their own strength that can be exploited

to improve the outcome of wound healing. A few examples of natural products are aloe vera (Pereira, Barrias, Granja, & Bartolo, 2013), gum arabic (Li et al., 2017), *Moringa oleifera* seeds (Parwani, Bhatnagar, Bhatnagar, Sharma, & Sharma, 2016), polysaccharides derivatives such as glucomannan (Zia, Zia, Zuber, Ahmad, & Muneer, 2016), berberine alkaloid (Xu et al., 2014) and honey (El-Kased, Amer, Attia, & Elmazar, 2017).

For honey-incorporated hydrogels, there are a few types of honey that have been used in the development of the hydrogels, such as Tualang honey, Manuka honey, Gelam honey (Imran, Dorai, Halim, & Sulaiman, 2010; Mohd Zohdi, Abu Bakar Zakaria, Yusof, Mohamed Mustapha, & Abdullah, 2012; Sasikala, Durai, & Rathinamoorthy, 2013). All these types of honey come from the *Apis Mellifera* species that are commonly known as the honeybee. However, there is another type of honey that comes from a stingless bee species (*Heterotrigona Itama* species). This honey is known as the stingless bee honey or in Malaysia and the Malay Archipelago, it is well-known as the Kelulut honey. The stingless bee honey contains a lot of wound healing properties such as a high amount of antioxidant, anti-microbial, anti-inflammatory for chronic inflammation, and has a good moisturizing effect (Abd Jalil, Kasmuri, & Hadi, 2017). Consequently, investigating the benefit of the incorporation of this stingless bee honey into the synthetic polymer would provide a promising effect on the outcome of the wound healing process.

## 1.2 PROBLEM STATEMENT

The application of honey on wound tissue has been practiced since ancient time due to its renowned healing properties. However, the use of raw honey in the form of solutions is not reliable as the tissue will absorb the fluid in a rapid manner. As a result, the dose of honey applied is not consistent. Besides, when it is combined with the traditional dressing such as gauze or bandage, it would cause discomfort to the patient due to frequent dressing change and might be painful during the dressing replacement (Boateng & Catanzano, 2015). Therefore, the introduction of honey incorporated with hydrogel would solve these problems due to its jelly-like structure, which is similar to the granulation tissue, thus reduces the pain and provides soothing effect (Phaechamud, Yodkhum, Charoenteeraboon, & Tabata, 2015). In addition, the hydrogel can allow a sustained delivery of the bioactive substances to the wound throughout time.

## 1.3 RESEARCH OBJECTIVE

### 1.3.1 General Objective

To develop a hydrogel film dressing containing stingless bee honey for effective wound treatment.

### 1.3.2 Specific Objectives

1. To evaluate the antimicrobial and immunomodulatory properties of the stingless bee honey.
2. To develop a hydrogel formulation and to incorporate stingless bee honey into the developed hydrogel formulation.

3. To characterize the physicochemical properties of the developed stingless bee honey-based hydrogel.
4. To determine the allergic reaction of the developed stingless bee honey-based hydrogel via acute dermal irritation test.
5. To determine the effect of the stingless bee honey-based hydrogel formulation on the wound healing process via histopathological assessments and wound contraction.

## 1.4 HYPOTHESIS

The stingless bee-based hydrogel treatment improves wound healing process.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 WOUNDS

A wound occurs when there is a disruption of skin integrity, mucosa surfaces, or organ tissue due to the internal pathological condition or external aetiology like mechanical, thermal, or chemical damage (Caló, Ballamy, & Vitaliy, 2018). Wounds can be classified into acute and chronic types based on the duration of the healing process.

Acute wounds usually take about three weeks to heal and include mostly epidermis and dermis layers (superficial wounds) but sometimes when the wounds involve subcutaneous layer, they are known as full-thickness wounds. The examples of acute wounds are abrasions, burns, laceration, tears, and chemical injuries (Dreifke, Jayasuriya, & Jayasuriya, 2015). Acute wounds can be caused by external aetiology such as mechanical damage due to stabbing by knives or incision during surgery. While thermal and chemical injuries occurred due to burning and other sources like radiation, electricity, and corrosive chemicals (Boateng & Catanzano, 2015). The burn is one of the worst forms of injuries that can be identified in three degrees. The first-degree burn involves epidermis only, second-degree burn involves a huge part of the dermis, and third-degree burn is where the skin is completely damaged (Shan et al., 2015).

Chronic wounds, on the other hand, take longer time to recover with a minimum of eight weeks and up to several years for severe cases. Several pathological conditions that cause these types of wounds are vascular or metabolic disorder from diabetes, inflammatory skin diseases, cancer, and persistent infection (Erfurt-Berge &

Renner, 2015). As a consequence, the wound healing is stuck in the healing cascade and failed to progress. This impairment causes excessive production of exudates that leads to maceration on the surrounding healthy tissues. Furthermore, the damage can extend to important tissues such as bones, joints, and nerves that may affect their physiological functions (Boateng & Catanzano, 2015; Gurtner, Werner, Barrandon, & Longaker, 2008). Some examples of chronic wounds are leg ulcers which include venous and ischemic types, diabetic foot ulcers, pressure ulcers, ulcerating cancer wounds, pyoderma gangrenosum wounds, and recently emerging buruli ulcer that is caused by mycobacterium infection (Bessis, Kempf, & Marsollier, 2015; Skórkowska-Telichowska, Czemplik, Kulma, & Szopa, 2013).

### 2.1.1 Wound Healing Mechanism

Wound healing is an outstanding biological process to restore skin integrity after injury. It can be divided into four overlapping phases which are haemostasis, inflammation, proliferation, and remodelling (Harper, Young, & McNaught, 2014; Jackson, Nesti, & Tuan, 2012). Figure 1.1 summarizes the interrelation of these phases.

#### *2.1.1.1 Haemostasis*

The immediate response of the body is to prevent uncontrolled blood loss and restore vascular integrity. Platelets aggregation occurs after they bind to von Willebrand factor (vWF) on vascular endothelium and gradually fills the vascular defect. Fibrin polymerisation is activated after coagulation cascades and then binds to platelets to form a clot. During the process, vasoconstriction causes the reduced blood flow which

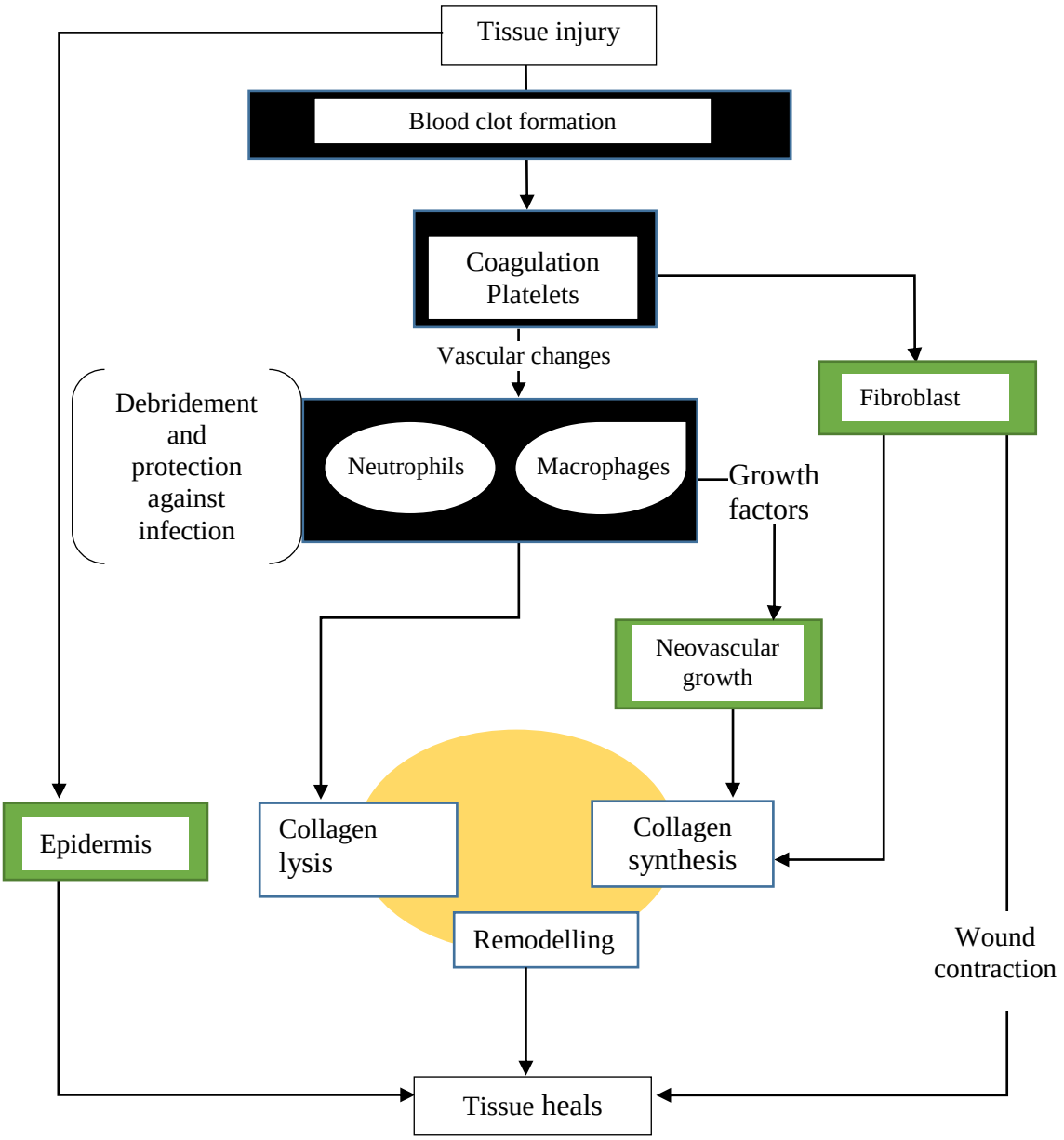


Figure 1.1 Fundamental interrelation of wound healing phases. ■ represents haematosis phase, ■ inflammation phase, ■ proliferation phase, and ● remodelling phase. Adapted from Boateng & Catanzano, (2015) and Harding et al., (2002).