รายงานวิจัยฉบับสมบูรณ์

โครงการวิจัย

การพัฒนาสูตรตำรับและการศึกษาฤทธิ์การสมานแผลของกรดไฮยาลูโรนิกและลิโดเคนบรรจุในสารประกอบเชิงซ้อนไคโตซานและเพคติน

Formulation development and Evaluation of Wound Healing Activity of Hyaluronic acid and Lidocaine Loaded in Chitosan-Pectin Polyelectrolyte Complex

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2562

ชื่อเรื่อง : การพัฒนาสูตรตำรับและการศึกษาฤทธิ์การสมานแผลของกรดไฮยาลูโรนิกและลิโดเคนบรรจุในสารประกอบเชิงซ้อนไคโตซานและเพคติน

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จำนวนหน้างานวิจัย: 25 หน้า

คำสำคัญ : สมานแผล กรดไฮยาลูโรนิก ลิโดเคน สารประกอบเชิงซ้อนไคโตซานและเพคติน

Key words : Wound Healing Hyaluronic Acid Lidocaine Chitosan-Pectin Polyelectrolyte Complex

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ผู้วิจัย : สุชารัตน์ ลิ้มสิทธิชัยกุล
สถาบัน : วิทยาลัยเภสัชศาสตร์ มหาวิทยาลัยรังสิต
ปีที่พิมพ์ : 2563
สถานที่พิมพ์ : มหาวิทยาลัยรังสิต
แหล่งที่เก็บรายงานการวิจัยฉบับสมบูรณ์: มหาวิทยาลัยรังสิต
จำนวนหน้างานวิจัย: 33 หน้า
คำสำคัญ : กรดไฮยาลูโรนิก, ลิโดเคน, สมานแผล, สารประกอบเชิงซ้อนไคโตซานและเพคติน
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บทคัดย่อ

การพัฒนาสูตรตำรับของกรดไฮยาลูโรนิกและลิโดเคนบรรจุในสารประกอบเชิงซ้อนไคโตซานและเพคติน ทำโดยการใช้โปรแกรม design expert เพื่อให้ได้สูตรที่แตกต่างกันของปริมาณกรดไฮยาลูโรนิกและลิโดเคนทั้งสิ้น 9 ตำรับ ส่วนประกอบที่แตกต่างกันของตำรับส่งผลต่อขนาดอนุภาค ค่าศักย์ซีต้า ร้อยการบรรจุของยา ร้อยละการปลดปล่อยของยา รวมถึงค่าความหนืดของตำรับ โดยพบว่า เมื่อเพิ่มปริมาณความเข้มข้นของกรดไฮยาลูโรนิก มีผลทำให้ขนาดอนุภาคเพิ่มขึ้น ค่าศักย์ซีต้ามีค่าติดลบเพิ่มขึ้น ร้อยละการบรรจุยาเพิ่มมากขึ้นและมีความหนืดที่เพิ่มขึ้นอย่างมีนัยสำคัญ ในขณะที่การเพิ่มปริมาณลิโดเคน มีผลทำให้ขนาดอนุภาคเพิ่มขึ้นและร้อยละการปลดปล่อยยามากขึ้น อย่างมีนัยสำคัญ ลักษณะของอนุภาคเมื่อทำการถ่ายภาพด้วยเครื่องอิเล็กตรอนแบบส่องกราด พบว่า มีลักษณะทรงกลมและมีพอลิเมอร์ล้อมรอบอนุภาค เมื่อนำไปทดสอบความเป็นพิษต่อเซลล์ พบว่า สารประกอบเชิงซ้อนไคโตซานและเพคตินที่ไม่มีลิโดเคน กรดไฮยาลูโรนิก ไม่มีผลต่อความเป็นพิษของเซลล์ร่างแหเหงือก ในขณะที่ลิโดเคนมีผลต่อการรอดชีวิตของเซลล์ร่างแหเหงือกตามความเข้มข้นที่เพิ่มขึ้น และพบว่า ที่ความเข้มข้นของลิโดเคน ที่ 50-100 ไมโครกรัมต่อมิลลิลิตร ไม่ส่งผลต่อการตายของเซลล์และยังช่วยเพิ่มปริมาณเซลล์อย่างมีนัยสำคัญ เมื่อทำการกรีดเซลล์ให้เกิดแผลและทดสอบด้วยลิโดเคนบรรจุในสารประกอบเชิงซ้อนไคโตซานและเพคตินความเข้มข้น 100 ไมโครกรัมต่อมิลลิลิตร เทียบกับ อาหารเลี้ยงเซลล์ สารประกอบเชิงซ้อนไคโตซานและเพคตินและสารละลายลิโดเคน ความเข้มข้น 100 ไมโครกรัมต่อมิลลิลิตร พบว่า ลิโดเคนบรรจุในสารประกอบเชิงซ้อนไคโตซานและเพคตินทำให้เซลล์มีการเคลื่อนที่มาปิดรอยกรีดในเวลาที่ 18 และ 24 ชั่วโมง ได้เร็วกว่า กลุ่มที่ทดสอบด้วยอาหารเลี้ยงเซลล์ สารประกอบเชิงซ้อนไคโตซานและเพคติน และสารละลายลิโดเคนอย่างมีนัยสำคัญ ตำรับกรดไฮยาลูโรนิกและลิโดเคนบรรจุในสารประกอบเชิงซ้อนไคโตซานและเพคตินมีฤทธิ์การสมานแผลในเซลล์และควรนำไปศึกษาเพิ่มเติมเพื่อพัฒนาตำรับที่มีคุณภาพต่อไป

Title : Formulation development and Evaluation of Wound Healing Activity of Hyaluronic acid and Lidocaine Loaded in Chitosan-Pectin Polyelectrolyte Complex
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Year of Publication : 2020
Publisher: Rangsit University
Sources: Rangsit University
No. of pages : 33 pages
Keywords: hyaluronic acid, lidocaine, wound healing, polyelectrolyte complex

Abstract

This study intents to develop hyaluronic acid and lidocaine loaded in chitosan-pectin polyelectrolyte complex by using design expert program and allocated 9 different hyaluronic acid and lidocaine formulations. Characteristic factors such as particle size, zeta potential, % of entrapment efficiency, viscosity and % of drug release were used to evaluate the formulations. The results showed that the more hyaluronic acid concentration, the bigger particle size, higher zeta potential, viscosity and %drug release increased. High concentration of lidocaine significantly increases the particle size and % of drug release. Microscopic characteristics of PECs solution, observed by TEM, appear as sphere-like vesicles embedded with polymeric micelles. Cytotoxicity test using human gingival fibroblast showed no cytotoxic of blank PECs, hyaluronic acid and other ingredients used while lidocaine affected to cell viability as a dose dependence manner. At concentration 50-100 µg/ml of lidocaine loaded in PECs found no cytotoxic but significantly help increasing cells proliferation. Cell scratch assay showed that 100 µg/ml of lidocaine loaded in PECs induced cellular movement and gap closer better than its blank PECs and lidocaine solution. Therefore, the hyaluronic acid and lidocaine loaded in PECs formulations which showed suitable in stability and drug release, found no cytotoxicity and improved wound closer should be carried out for better effectiveness and should be evaluated for further studies.

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Chapter 1
Introduction

Background and rationale

Patients with oral surgery wound, commonly occurs in the dental practices, generally suffers with pain and postoperative wound such as swelling and inflammation which sometime can cause infection. Although the wound can heal naturally as defensive mechanism of the body, failure acute management can cause problems and leads to become chronic wound or severe infection. Thus, primary outcome for treating oral wound surgery is pain reduction followed by enhaning wound healing activity.

Four basic phases of wound healing are the hemostatic phase, inflammatory phase, proliferative phase and remodeling phase (Schultz, Chin, Moldawer, & Diegelmann, 2011). Primary wound healing is an ideal for healing of wound because primary closure has a small, clean defect that minimizes the risk of infection and requires new blood vessels and fibroblast can migrate in a small distance. Secondary wound healing requires a granulation tissue to fill the wound defect. This type of closure requires more time and energy than primary wound closure and creates more scar tissue. Several of surgical wound cannot always make primary closure such as donner site of mucosal graft, extraction wound, dehiscence complication postoperative pain and swelling. Type of flap such as primary closure in surgical removal of impacted tooth increase pain and swelling more than secondary wound healing (Pasqualini, Cocero, Castella, Mela, & Bracco, 2005). The dentists must to supportive treatment for relief this problem for example cold pack for control swelling, systemic analgesic for relieving pain and appoint for manage in delay wound healing.

Drug delivery systems in wound healing that release antimicrobial and anti-inflammatory drugs represent a great opportunity to prevent infections or enhance the effectiveness of current commercial drugs. The study aims to prepare and evaluate whether chitosan could be used to prepare wound healing product which would lead to suggest further therapeutic options for treating wound healing. Design and optimization would be used to carry out the suitable formulation along with physicochemical properties and stability of the formulations. Wound healing activities would be performed in cell culture methods to find out cytotoxicity and wound closure effects.

Objectives

The main objective of this study is to develop formulation composed of chitosan and pectin incorporated with hyaluronic acid and lidocaine HCl for the uses as a topical pain reducer and wound healing product. To achieve the main objective, minor objectives of this study are followed by

a) to design and optimize the formulation composed of chitosan-pectin-hyaluronic acid and lidociane HCl in order to find out the suitable formulation.

b) to evaluate the formulations that provide safety and express wound healing activity.

c) to investigate and evaluate the physicochemical properties and stability of the formulations containing hyaluronic acid and lidocaine HCl formulation

Research hypothesis/research questions

1. Dose polyelectrolyte complexes composed of chitosan and pectin help deliver hyaluronic acid and lidocaine HCl to the cells and induce the wound gap and wound closure in lower concentration than standard uses?
2. Dose polyelectrolyte complexes composed of chitosan and pectin with hyaluronic acid and lidocaine HCl induce the wound gap and wound closure faster than using hyaluronic acid and lidocaine HCl alone?

Scope of research

This study aims to prepare and evaluate whether the chitosan-pectin polyelectrolyte complexs containing hyaluronic acid and lidocaine HCl could be formulated to be used as pain reducer and wound healing formulation which would lead to suggest further therapeutic options for treating wound healing. Design and optimization would be used to carry out the suitable formulation along with physicochemical properties and stability of the formulations. Wound healing activities would be performed in cell culture methods to find out cytotoxicity and wound closure effects.

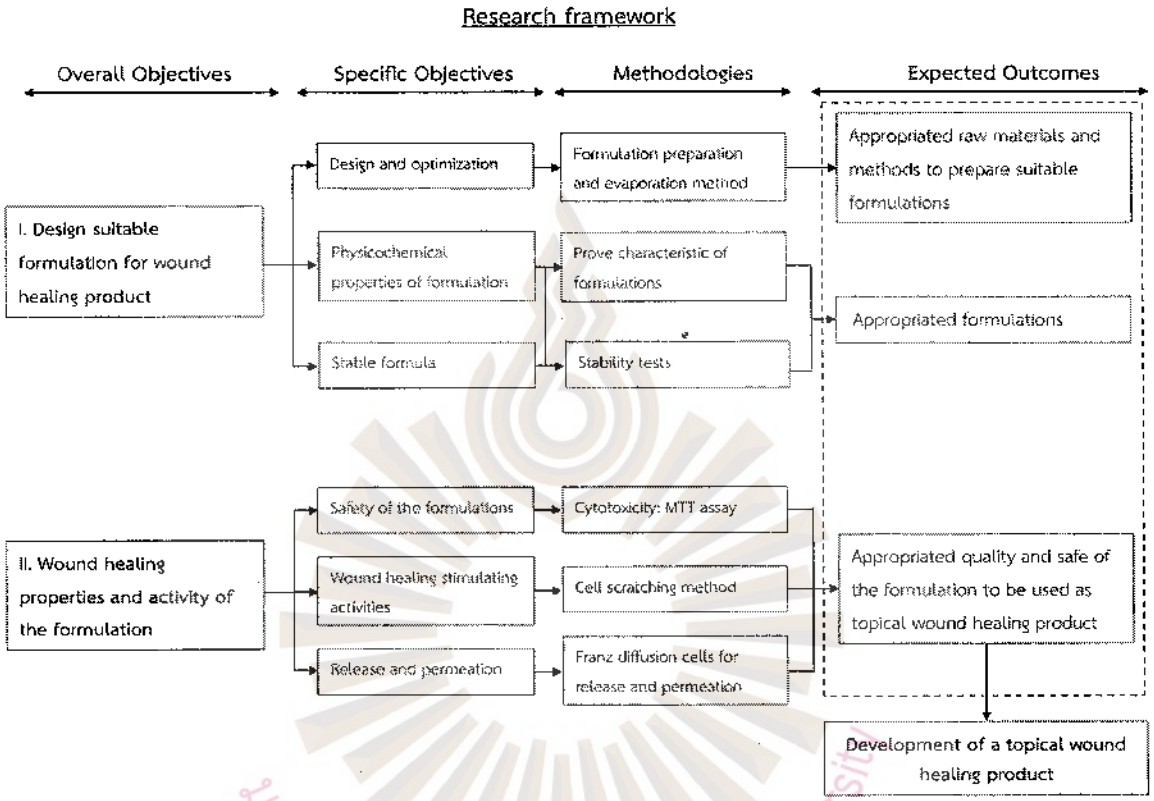
Duration of research (ระยะเวลาที่ทำวิจัย)

12 เดือน

Conceptual framework (กรอบแนวคิดในการวิจัย)

The research will be divided into 2 main parts

1. Design suitable formulation for wound healing product
2. Wound healing properties and activity of the formulation



Beneficial of research

1. Suitable formulation for relieving pain and promoting wound healing from surgical wound in oral cavity
2. Reduction of the drug concentration which would decrease the side effect of the drug.
3. Further study in animal and clinical trials for the uses in oral surgery wound or other dental wound problems such as dry socket wound, periodontal diseases and/or other dental wounds.
4. The suitable formulation will apply for the patent.

Chapter 2
Review literature

Wound healing

Wound healing is a natural body function by which the body repairs itself after injury. The primary objective of the healing process is to fill the gap created by tissue destruction followed by restoration of the structural continuity of injured part via 3 phases of healing: the inflammation phase, proliferation phase, and remodeling phase.

1.1 Inflammation phase

First, inflammation aims primary at removing the injury causing agent and letting the extent of tissue damage as it prepares the wound environment for healing. Inflammation is manifested by redness, welling, heat, pain, and loss of function. The phase begins with arterioles and veins near the site of injury constricting briefly, then the vessels dilate promoting congestion and accompanying increases in capillary permeability leads to the movement of fluid into the effected tissues. Increasing viscosity causes the blood to flow more slowly and clotting occurs. Phagocytic white blood cells or leukocytes emigrate trough the vessel walls into the inflamed tissue where the leukocytes engulf and degrade the bacteria and cellular debris in the process call phagocytosis. Phagocytosis is part of the immune mechanism to prevent an infection that would impair wound healing. Subsequently, the release of growth factors leads to the attraction of fibroblast.

1.2 Proliferation phase

The arrivals of fibroblasts mark the beginning of the second phase of wound healing call the proliferation phase. In this phase is focus on the building of new tissue to fill the wound space. Fibroblasts are connective tissue cells that synthesize and secrete collagen. They also secrete growth factor that induces the growth of blood vessels though the process call angiogenesis while promoting endothelium cell proliferation and migration. One of the growth factors is vascular endothelium growth factor (VEGF). VEGF is a growth factor that is the most prevalent, efficacious, and long-term signal known to stimulate angiogenesis in wounds enhances migration and proliferation of endothelial cells and induces vascular permeability consistent with the purported ability to promote angiogenesis. The fibroblast and endothelium cell forms

granulation tissues that serve as the foundation for scar tissue development. Granulation tissue contains newly developed capillary bloods. The tissue is soft and pain because it is fragile granulation tissue bleeds easily. The newly formed blood vessels are leaking and allow plasma proteins and white blood cells to leak into the tissues. The final component of the proliferative phase is epithelialization which is the regeneration migration proliferation and differentiation of epithelium cell. At the wound adequate form a new surface area similar to that destroyed by the injury. By the end of this phase, white blood cell leave the wound site edema diminish, and the wound begins to branch.

1.3 Remodeling phase

The third phase of wound healing is the remodeling phase. It begins after about 3 weeks and can continue for 6 months or longer. During this stage final scar tissue is being formed by simultaneous synthesis and line with collagen. Clinically, the scar becomes a vascular. Scar tissues may achieve 70-80% of counsel strength by the end of 3 months.

Oral surgery wound and treatment

Oral surgery wound in dentistry commonly occur in routine clinical practice. Even though oral surgical wounds heal in a self-defensive pathway, untreated or failure acute management can cause problems and leads to become chronic wound or bacterial infection. Normally this oral surgical wound healing manages by using anti-bacterial dressings such as clindamycin, Metronidazole, chlorhexidine, or applying obtundent dressings such as Eugenol. However, severe surgical wound especially in periodontal and implant dentistry has to be monitored and used specific pain killer to help relieve the pain.

The treatment of the oral surgery wounds depends on each dentist's experience mainly due to the complex etiology. Pain is a primary action that needs to be solve. Usually topical anesthetic dressings such as Lidocaine, Oraqi gel have been apply. However, the problem is that those anesthetics have a short action and duration.

Nanoparticle based delivery systems in wound healing

Drug delivery systems in wound healing that release antimicrobial and anti-inflammatory compounds represent a great opportunity to prevent infections or enhance the effectiveness of current commercial drugs. Many biocompatible biomaterials have been extensively investigated

to deliver drugs into wound beds and to improve wound healing. Nanoparticles approaches that employ materials engineered with at least one dimension within the nanoscale (1–100 nm) were pioneered to efficiently control wound healing and minimize any possible complication that might surface during this process. There are two main categories of nanomaterials used in wound healing; a.) nanoparticles that exhibit intrinsic properties beneficial for wound treatment and b.) nanoparticles employed as delivery vehicles for therapeutic agents. The major advantage of nanoparticles over their bulk counterparts is the versatility and tunability of the physicochemical properties. Furthermore, the high surface area to volume ratio endows nanostructures with unique features. Nanoscale particles provide for a high probability of interaction with the biological target and an enhanced penetration into the wound site. As a result, nanoparticles have an ability to deliver a sustained and controlled release of therapeutics those results in an accelerated healing process.

Nanoparticles based delivery systems gained widespread recognition because of their promising potential and advantages over the conventional approaches. These advantages include

a.) avoidance of biodegradation of the encapsulated cargo,

b.) facilitation of the controlled/sustained release of the encapsulated drugs,

c.) enhancement of dissolution rate and permeability of the poorly water-soluble drugs,

d.) prolongation of plasma half-life and improved pharmacokinetic profile of the drugs,

e.) improvement of cellular uptake for efficient targeting of bioactive molecules, and

f.) optimization of target-specific delivery of drugs and superior drug retention into the target tissues. The nanoparticles-based attempts include polymeric micelles, vesicular delivery systems, hydrogel, nanoemulsion, nanohybrid scaffolds, nanostructured lipid carriers (NLCs), nanofibrous structures and polymeric nanoparticles. Polymeric nanoparticles have been studied by many researchers to evaluate their ability to improve pharmaceutical significance and therapeutic viability of the encapsulated drugs. The success of this nanocarrier delivery system is due to their ultra-small size, high encapsulation efficiency, optimum zeta potential, biodegradability and biocompatibility.

Nanoparticles have been explored to deliver silver and morphine to chronic wounds, to inhibit bacterial colonization, and to reduce pain. In addition, nanoparticles have been used to encapsulate and protect therapeutic agents from degradation in the highly proteolytic wound

environment. Vasoactive agents, such as nitric oxide, have also been stabilized and released using nanoparticle technologies to prevent or treat ischemia. Recently, Chigurupati et al. reported on the topical use of cerium oxide nanoparticles for healing full-thickness dermal wounds in mice via a mechanism that enhances the proliferation and migration of various skin cells. The results suggest that cerium nanoparticles penetrated into the wound tissue and reduced oxidative damage to cellular membranes and proteins.

Natural polymeric nanoparticles

Natural polymeric nanoparticles such as chitosan-based nanoparticles have been the most extensively studied for topical drug delivery applications. Chitosan is a linear polysaccharide derived from chitin, which is found in the exoskeleton of crustaceans. The amino group of chitosan readily undergoes protonation in acidic to neutral conditions, resulting in a net positive charge. This makes chitosan water- soluble and act as a bioadhesive, since the positively charged chitosan can bind to negatively charged mucoproteins. This interaction helps prolong the circulation time of chitosan-bound drugs, leading to enhanced drug bioavailability. Chitosan nanoparticles can be prepared using (i) ionic cross-linking, (ii) covalent cross-linking, (iii) precipitation, (iv) polymerization, or (v) self-assembly methods with sizes ranging from 20 to 800 nm dependent on the method of preparation. Chitosan-drug complexes are generally formed via electrostatic interactions between cationic chitosan and anionic drugs.

The use of biocompatible, absorbable polymers such as pectin, chitosan, gelatin and hyaluronic acid having a unique set of biological properties including biocompatibility, biodegradability and low to absent toxicity. Chitosan has been found to be an attractive material for wound healing applications. In addition, chitosan has antibacterial, hemostatic and mucoadhesive properties and may act as a wound healing accelerator. Chitosan and its derivatives were used for skin and wound healing in gels, micro- or nanoparticles and films. Chitosan is a unique biodegradable, biocompatible polymer possessing hemostatic properties which make it a prospective candidate in the wound healing substrates. It could be applied in wound healing sponge scaffolds which should be non-toxic, non-allergic and allow proper nutrient and gas exchange. Due to their well- interconnected microporous structure, chitosan sponges have good cell interaction, fluid absorption capability, and hydrophilicity. Unfortunately, the high brittleness

of chitosan is the most pronounced inadequacy in formulating wound healing materials, which could be overcome by blending them with other polymers.

Chitosan-based wound dressings possess a set of unique properties, including hemostatic, biodegradable and antibacterial properties that make them useful for wound healing. Antibacterial activity of chitosan was already observed at low concentrations against a variety of pathogens such as *Escherichia coli* or *Staphylococcus aureus* and may be used in various formulation types. Chitosan nanoparticles can be produced due to electrostatic interactions with a negatively charged lipid such as lecithin, as the ones produced in a study by Blazevic et, al. Those chitosan/lecithin nanoparticles were loaded with melatonin, and their efficacy was demonstrated by their ability to promote wound healing *in vitro*.

Cross-link between chitosan and other polymers and/or polysaccharides showed progress effects in wound healing product development

The effect of chitosan as an accelerator of wound healing from

1. Effect on PMN Chitosan can promote wound-healing by enhances inflammatory cells functions such as macrophages, polymorphonuclear leukocytes and fibroblasts.

2. Effect on promotes granulation Chitosan promote granulation tissue in the large open wounds of animals(Ueno et al., 1999)(Ueno, Mori, & Fujinaga, 2001), The effects of chitin and derivatives of chitin on the proliferation of fibroblasts was showed in vitro. However, the inhibition of cell proliferation found in high concentrations of chitosan that was not shown in cultures without FCS.(Mori et al., 1997).

Pectin is a natural poly anion(Morris et al., 2016), heterogeneous class of negatively charged polysaccharides derived from plant cell walls, containing galacturonic acid which can be partially methyl esterified. Pectin has anti-inflammatory activity from galacturonic acid content (Markov, Popov, Nikitina, Ovodova, & Ovodov, 2011). The strong anti-inflammatory activity is due to Pec's high level of esterified galacturonic acid residues which suppress of iNOS and COX-2, two of the most important enzymes in the inflammatory process. Pectin has recently been investigated for use in various biomedical applications including drug delivery, chronic wound management.

Hyaluronic acid (HA) is a component of the extracellular matrix of high molecular weight glycosaminoglycan. They found in high concentrations in many tissues such as soft connective

tissues, skin, umbilical cord and synovial fluid. HA have biocompatibility, Anti-inflammation, Anti-edematous and Antioxidant (Mani et al., 2016)

Lidocaine HCl is a widely of local anesthetic drug. Lidocaine is an effective and reliable hydrophilic local anesthetic, which has been widely used in the dental treatment of early onset topical anesthesia Topical used in lidocaine HCl have many forms for example spray, gel and patch. Early onset is a good property to relief pain

Chapter 3
Methodology

1. Preparation method

1.1 Preparation of chitosan-pectin polyelectrolyte complexes (PECs)

The cross-linkers are usually employed to obtain chitosan and pectin particles. The ionic cross-linkers that cross-link through ionic gelation and electrostatic interactions between the positively charged chitosan chains, and the mixture of polyanions, pectin was added dropwise to chitosan solution with stirring. The polyelectrolyte complexes between chitosan and pectin were observed their turbidity by UV-VIS spectroscopy.

1.2 Preparation of hyaluronic acid and lidocaine into the CS/PT PECs

The ionic gelation cross linkage of the PECs and hyaluronic acid is carried out by adding hyaluronic acid solution while stirring. Then, the lidocaine HCl is added with homogenized. After stirring, each opalescent solution was centrifuged and the amounts of the free drug in each supernatant were measured in order to analyze the entrapment efficiency.

2. Characterization

2.1 Particle size and zeta potential

The particle size, size distribution (polydispersity index; PDI) and zeta potential of PECs particles were measured by Zetasizer, based on the dynamic light scattering (DLS) technique.

2.2 Morphological observations

Examinations of surface morphology and size distribution for the PECs were performed using a transmission electron microscope (TEM).

2.3 Entrapment efficiency

In order to determine the entrapment efficiency of the PECs, it was necessary to detect the amount of free drug in the clear supernatant. The drug entrapment efficiency was calculated using the following equation (Eq. 1):

$$\text{Entrapment efficiency \%} = \frac{(\text{Total drug used in formulation} - \text{Free amount of drug}) \times 100}{(\text{Total drug used in formulation})} \quad \text{(Eq. 1)}$$

2.5 Stability

Preliminary of the PECs formulation stability was done by following Guidance for Industry: Bioanalytical Method Validation. The criteria of choosing formulation that undergoes stability test was followed such as still in appearance and consistency of the formulation based without separation or aggregation of solution, color, etc. The PECs preparations were kept at 3 different temperatures at room temperature and observed for it appearance after freshly prepared, 1 day after prepared and 3 month after storage. At a time of stability testing, the samples were randomly collected and evaluated physical parameters. The residue quantity in each time was led to degradation rate constant and finally stability of the formulations.

3. *In vitro* cytotoxicity studies

3.1 Cells preparation

The cytotoxicity test would be performed by following the OECD guideline. Fibroblast were grown in 75-cm^3 tissue culture flasks in complete media (10%FBS and 90%DMEM and 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 25 µg/ml of amphotericin B, in humidified CO_2 incubator containing5% CO_2 at 37°C. When the cells grow and/or are ready to use, they were trypsinized by 0.25% trypsin/EDTA solution. The cells were seeded into a 96 well plate for treating in the experiment. In each test using a 96 well plate at about 10,000 cells/well.

3.2 MTT assay for cytotoxicity

MTT assay was performed by incubating the cells cultured in 96-well plates using CO_2 incubator at 37°C. After 24 hours, the plated cells were treated with the PECs formulations for 24 h. After the incubation period of 24 hours, the medium was removed from the well-plate and replaced by adding 0.5 mg/ml of MTT and incubating for 3 hours. Colorimetric detection of living cells was determined as violet color while dead cells remain original yellow color of MTT solution. Then, MTT solution was removed and added DMSO solution and measured the absorbance at 550 nm by using a microplate reader. The results are used to calculate cell viability by comparison to positive (1%H_2O_2) and negative control (media) which would be treated in the same well plates. The percentage of cell viability (%) was calculated using the following equation (Eq. 2):

$$\text{Percentage of cell viability (\%)} = \frac{\text{(Mean absorbance of sample)} \times 100}{\text{(Mean absorbance of negative control)}} \quad \text{(Eq. 2}$$

3.3 Cells scratching and gap measurement

Cell migration and cell invasion were presented as a percentage (%) of cell motility which is referred to as healing. HFF cells concentration of 2,000,000 cells/well was seeded into a petri dish 100 mm. When the cells reached to 80% confluence (monolayer), a scratch wound was done by using a sterile pipette tip, then adding the samples in various concentrations was added and cell proliferation at 24 hours was measured. DMEM without FBS was used as the negative control. Zinc sulphate solution (2 µM) and 20 % (v/v) FBS in DMEM medium solution were used as positive controls.

Gap of scratched cells by 200 µl pipette tip were observed by 50X Inverted microscope (10X of objective lens and 5X of eyepiece lens, Olympus, Japan) with digital camera (ZEISS Axio Vert.A, U.S.A.) and image proplus 7.0 (Image Processing Software, U.S.A.) was used to measure the gap area of HFF cells at time 0, 18, and 24 h. The scale of measurement was calibrated before measuring.

4. *In vitro* release study

In vitro release study using vertical Franz diffusion cells mounted by a sheet of cellulose membrane (pore size 0.02 µm) as the barrier between the sample as the donor and artificial saliva pH 7.4 as the receptor at 37°C, 600 rpm. Sink condition was maintained by withdrawal of the testing receptor medium and immediate replacement with an equal volume of fresh medium. The samples of the receptor medium were quantitated by UV spectroscopy for released samples from the formulation.

5. Statistical analysis

The optimization of all formulations would be expressed as mean ± standard deviation (SD). Coefficients of variation (CV) were calculated for determination of precision of data and methods. Statistical significance was determined by one-way analysis of variance (ANOVA). The significant level was considered at $p < 0.05$. Statistical analysis of the experimental data will be carried out using SPSS statistic program version 19.0.

Chapter 4

Results and discussion

PECs characteristics

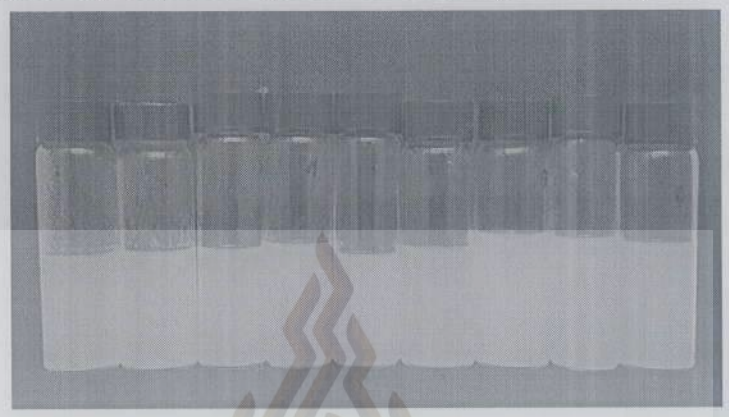
Factorial design of polyelectrolyte complexes composed of chitosan and pectin with hyaluronic acid and lidocaine HCl was performed 2 factors 3 level full factorial design and assessed 9 different formulations which varied the ratio of lidocaine HCl from 4-10% and hyaluronic acid from 0.5-1.5% according to the range of lidocaine and hyaluronic dose concentration as shown in table 1.

Table 1 The 2 factors 3 level full factorial design of Lidocaine HCl (LD) and hyaluronic acid (HA) incorporated in polyelectrolyte complexes composed of chitosan and pectin to assessed 9 different formulations following factors as particle size, zeta potential, %entrapment efficiency (%EE), drug release at 5 min, and viscosity (cP).

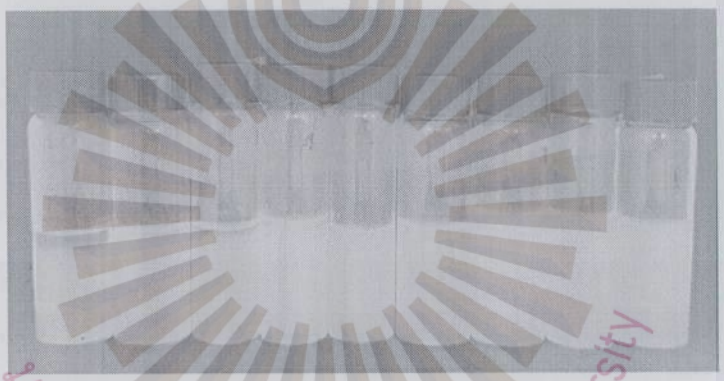
Rx	Lidocaine HCl (X1)	Hyaluronic acid (X2)	Size (μm)	Zeta (mV)	%EE	Drug releasing (%)	Viscosity (cP)
LD 1	-1	-1	2.25	-21.42	38.39	41.99	5.4
LD 2	0	-1	1.31	-9.56	45.90	20.80	8.8
LD 3	1	-1	3.58	-6.76	40.51	12.65	14.4
LD 4	-1	0	2.13	-20.76	73.05	43.16	26.8
LD 5	0	0	8.42	-22.35	74.79	21.40	32.0
LD 6	1	0	4.06	-11.61	74.25	15.00	57.0
LD 7	-1	1	4.83	-27.66	76.31	52.29	177.2
LD 8	0	1	7.08	-17.04	86.72	24.08	273.4
LD 9	1	1	4.17	-23.34	92.33	15.89	210.8

Stability

All formulations are shown to be white turbid solution after prepared as shown in Fig. 1A. However, formulation LD 1, 2, and 3 starts to separate and clear to see in separation 1 day after prepared. After 3 months will found LD 4, 5 and 6 found separated (Fig. 1B).



(A) freshly prepared



(B) 1 day after prepared

(C) 3 mos. after prepared

Figure 1 Stability of all formulations as (A) freshly prepared (b) 1 day (c) 3 months after storage.

Particle size and zeta potential

The particle size of the PECs in the range of 2.0-4.6 μm and formulation LD 7, 8 and 9 provided the largest size than others. The anionic-cationic charge ratio of formulation is an effect on the formation of polyelectrolyte complex. All the formulations are the negative zeta potential value from a high concentration of negative charge in formulation and zeta potential was measured in the range of -6.9- (-27.66) mV. (Fig. 2 and table 1)

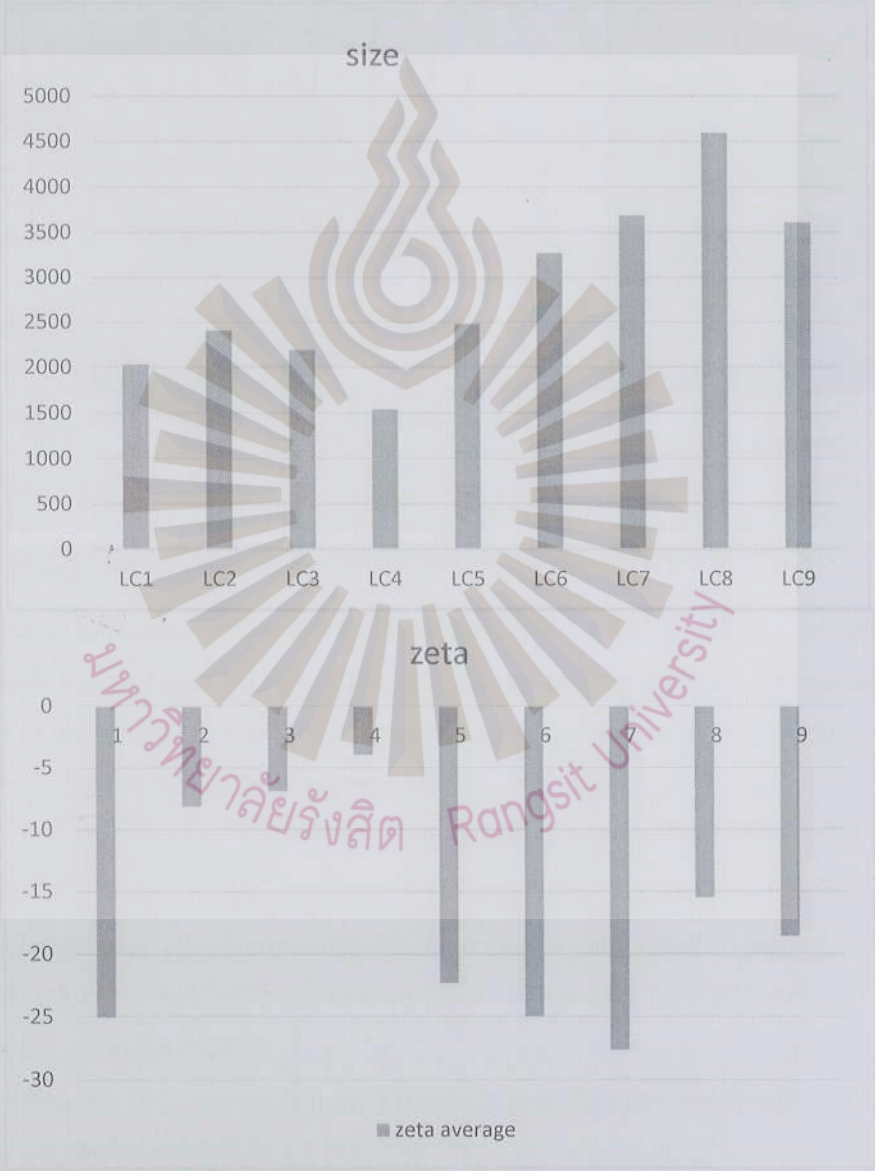


Figure 2 size and zeta of all 9 formulations

Entrapment efficiency

Entrapment result from 2 factors 3 level full factorial design was showed in table 1. From the design, we get entrapment efficiency as (R^2 = 0.9978)

Entrapment efficiency = $-2.06A^2-9.4B^2+5.37AB+1.96A+20.49B+75.41$

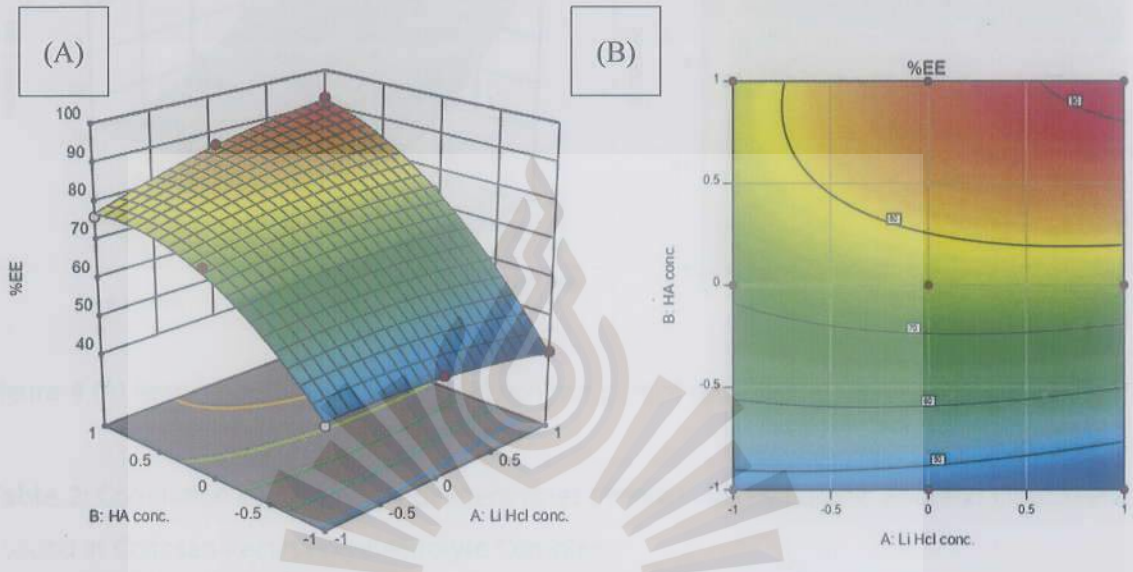


Figure 3 (A) Response surface plot and (B) contour plot for entrapment efficiency

The range of entrapment in this study was detected is 40.51-92.33%. The formulation that uses a high concentration of hyaluronic acid was presented with high entrapment. Because of the effect of viscosity to formulation represent to entrapment of drug, in Table 1, show high viscosity formulation have high of entrapment drug.

Drug release

The objectives of drug formulation for dry socket is early onset in pain killer, therefore burst effect in lidocaine hydrochloride release is very important. Drug releasing in 5 min is a one of goal for this preparation.

Drug releasing at 5 min result from 2 factors 3 level full factorial design was showed: Drug release = $8.24A^2-1.1B^2-1.76AB-15.48A+2.8B+21.36$ (R2 = 0.9910)

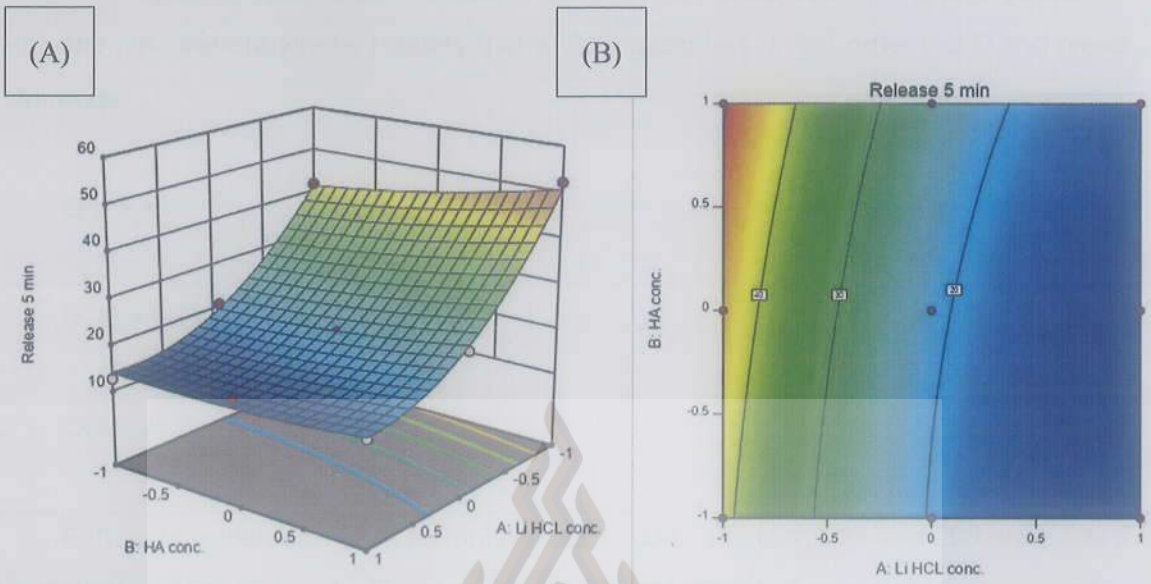


Figure 4 (A) Response surface plot and (B) contour plot of drug release at 5 min.

Table 2: Correlation coefficients of different kinetic models for Hyaluronic acid and Lidocaine Loaded in Chitosan-Pectin Polyelectrolyte Complex

Rx	Zero order			First order			Higuchi		
	r2	slope	Intercept	r2	slope	Intercept	r2	slope	Intercept
LD1	0.9982	35.488	39.973	0.9706	0.2154	1.6337	0.9781	60.892	19.808
LD2	0.9615	37.605	23.129	0.8593	0.3171	1.4112	0.9976	66.396	0.4129
LD3	0.9814	40.157	23.129	0.8726	0.4102	1.32238	0.9934	69.856	10.769
LD4	0.993	35.585	43.15	0.9533	0.2082	1.6618	0.9888	61.55	22.575
LD5	0.9781	39.648	19.924	0.8976	0.3396	1.3714	0.9901	69.146	3.4092
LD6	0.9889	43.803	15.815	0.8688	0.3878	1.3057	0.9869	75.853	9.575
LD7	0.993	33.373	49.871	0.9684	0.1816	1.7184	0.9803	57.477	30.753
LD8	0.9951	39.021	20.637	0.9427	0.3208	1.238	0.9766	67.005	1.5733
LD9	0.9961	36.218	13.028	0.9355	0.375	1.238	0.9765	62.159	7.5636

From Table 2, %cumulative amount of Lidocaine HCl for 120 min releasing were examination in different kinetic models that is Zero order (eq.2), first order (eq.3) and higuchi (eq.4) model

$Q = k_0 t$ (eq.2)

$\ln Q = \ln Q_0 - k_1 t$ (eq.3)

$Q = k_H t^{1/2}$ (eq4)

Follow consideration of R^2 formulation LD1, LD4, LD6, LD7, LD8 and LD9 were fitted to Zero order and LD2, LD3 and LD5 were fitted in higuchi model. Zero order kinetic model is not depending on the concentration or surround (environment) situation that has various of advantages for apply to drug treatment (Tang et al., 2015).

From all results of design, we found that concentration of lidocaine HCl affects to the release and particle size while the concentration of hyaluronic acid affects to viscosity and stability of the PECs solution. Therefore, formulation LD3, LD6, LD9 were selected for further study.

Morphological observations

Microscopic characteristics of PECs solution were observed by TEM (Fig. 6) appear as sphere-like vesicles.

Cell viability

The cell viability was evaluated by varies concentrations, selected formulation LD3, LD6 and LD9 were chosen form data about %EE, drug release and stability. All composition of PECs such as hyaluronic acid, CS/PT, and blank PECs did not affect to the cell viability. However, LD3, LD6 and LD9 and lidocaine HCl solution at concentration 250-1000 µg/ml decreased the cell viability about 20% compared to the negative control while the concentration of lidocaine at 50-100 µg/ml did not showed cytotoxicity but improve cell proliferation as shown in Fig.7 and 8.

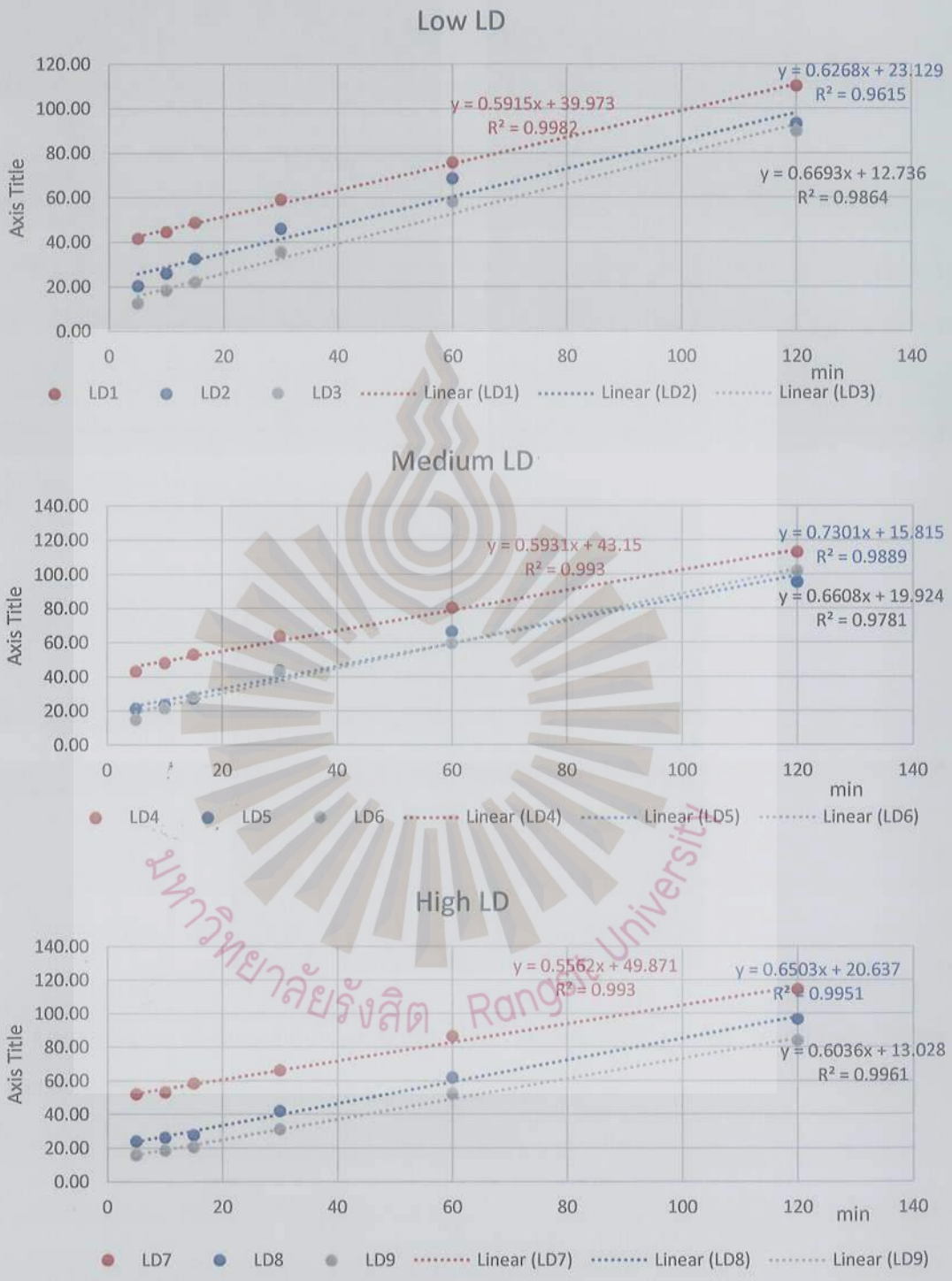


Figure 5 *In vitro* release of all PECs formulations which showed the release pattern by varying hyaluronic concentration and low, medium and high concentration of lidocaine.

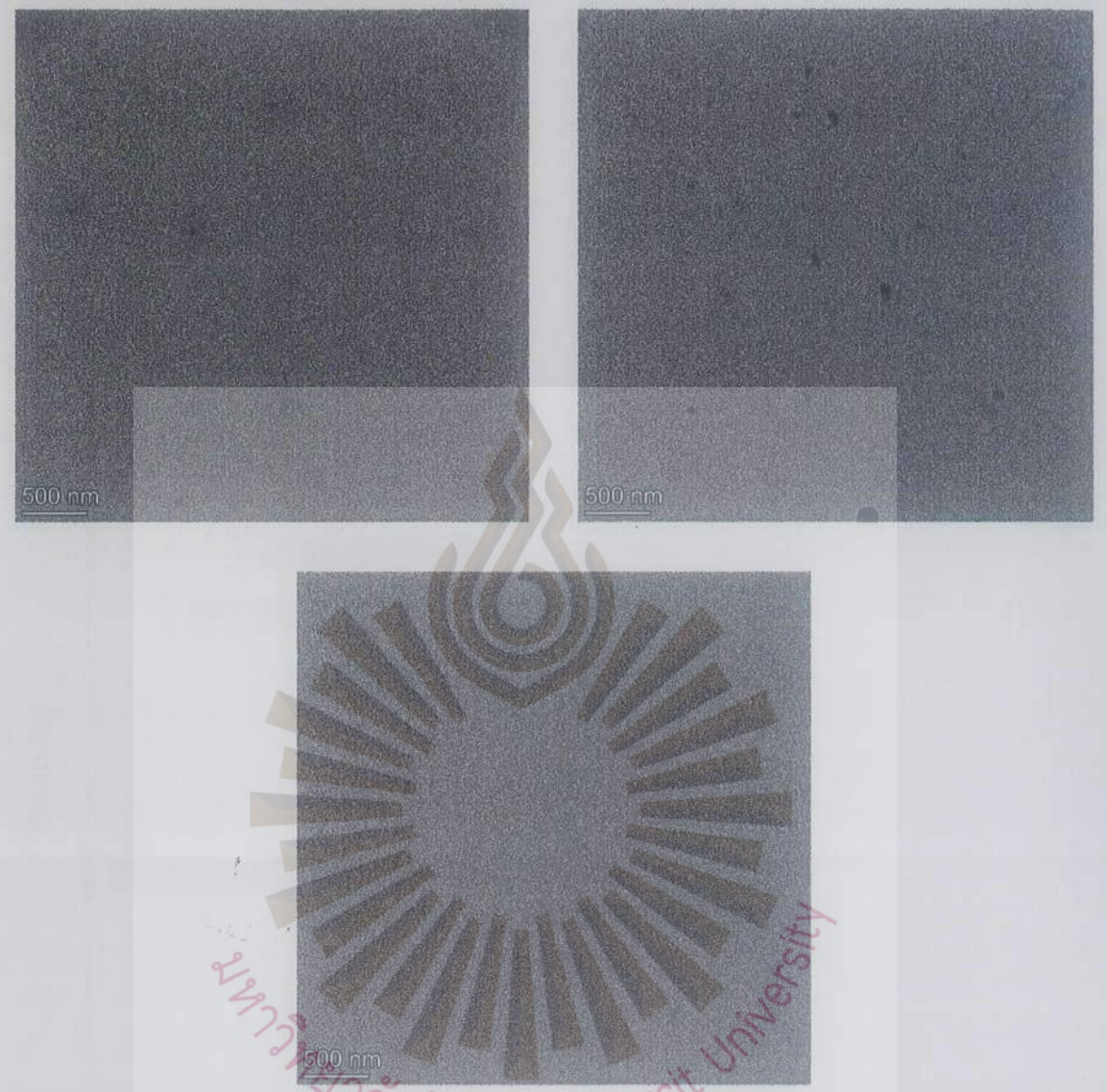


Figure 6 Micrographs observed through transmission electron microscopy (TEM) of (A) LD2 (B) LD6 and (C) Ld9

Figure 7 Viability determined by MTT

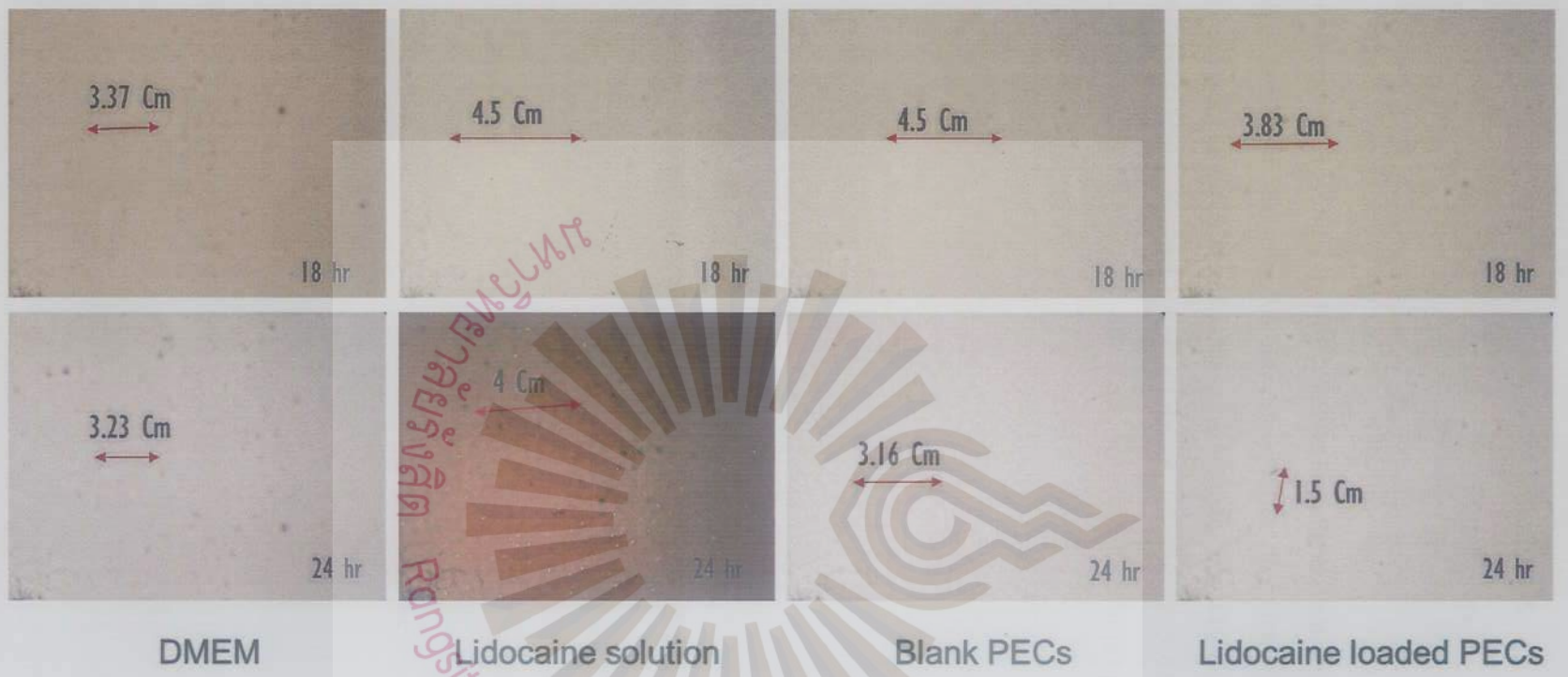


Figure 8 Scratch wound of HGF treated with DMEM solution, lidocaine solution, blank PECs and lidocaine loaded PECs at 18 and 24 h.

Chapter 5
Conclusion

The most importance to develop the chitosan-pectin polyelectrolyte complexes formulation is to find out the suitable ingredient that provide the most stable formula, great characterization and improve drug delivery or drug activity. The PECs were introduced to help delivery lidocaine for the use of wound healing. The lidocaine loaded PECs provided appropriated characterization, showed suitable release, fitted in zero order model, expressed no cytotoxicity and showed good stability which supposed to be the proper formulation for the future topical oral wound healing uses.

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ปรด	วิจัยและพัฒนาเภสัชภัณฑ์	เภสัชศาสตร์	มหาวิทยาลัยขอนแก่น	2559
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1. Research and development in pharmaceutical sciences
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