Preparation of Chitosan Films and Microspheres by Cross-linking Reaction Using De-proteinated Epoxidized Natural Rubber for Skin Drug Delivery

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ชื่อวิทยานิพนธ์ การเตรียมฟิล์มและเม็ดไคโตแซนด้วยปฏิกิริยาครอสลิงค์กับน้ำยางธรรมชาติที่มีหมู่อีพอกไซด์ปราศจากโปรตีนเพื่อใช้ในการนำส่งยาทางผิวหนัง

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บทคัดย่อ

ฟิล์มไคโตแซนสามารถเตรียมได้สำเร็จโดยปฏิกิริยาคลอสลิงค์โดยใช้ยางธรรมชาติอิพ็อกซิไดซ์ ในการศึกษานี้ยางธรรมชาติอิพ็อกซิไดซ์ที่มีหมู่อิพ็อกไซด์ร้อยละ 25 (ENR-25) และร้อยละ 50 (ENR-50) ในปฏิกิริยาคลอสลิงค์ถูกกำจัดโปรตีนโดยกระบวนการสกัดก่อนนำมาใช้ในการศึกษาต่อไป สูตรที่ใช้ในการเตรียมฟิล์มไคโตแซนประกอบด้วย 1.5% w/v ไคโตแซน (ขนาดโมเลกุลเฉลี่ย 300 กิโลดาลตัน) ใน 1% กรดอะเซติก ปริมาตร 100 มิลลิลิตร นำมาทำปฏิกิริยาคลอสลิงค์กับยางธรรมชาติอิพ็อกซิไดซ์ปราศจากโปรตีน โดยในกรณีของยางธรรมชาติอิพ็อกซิไดซ์ปราศจากโปรตีนร้อยละ 25 ใช้ 1 mL ของ 4% w/v และใช้ยางธรรมชาติอิพ็อกซิไดซ์ปราศจากโปรตีนร้อยละ 50 ใช้ 1 mL ของ 1% w/v ที่ละลายในเตตระไฮโดรฟิวแรน ไคโตแซนฟิล์มได้มาด้วยการทำให้แห้งที่อุณหภูมิ 40 องศาเซลเซียส ผลการศึกษาพบว่าไคโตแซนฟิล์มที่ได้มีคุณสมบัติทางเชิงกลที่ดีมาก เช่น คุณสมบัติการพองตัว การดูดความชื้นที่สูง ความเค้นดึงสูงสุดเพิ่มขึ้น และร้อยละการยืดของไคโตแซนฟิล์มที่จุดขาดสูงขึ้น ไคโตแซนฟิล์มที่เตรียมได้ไม่ก่อการแพ้แก่ผิวหนัง ปฏิกิริยาคลอสลิงค์เกิดโดยปฏิกิริยาการเปิดวงด้วยนิวคลิโอไฟด์ของวงอิพ็อกไซด์ซึ่งทำให้เกิดโครงสร้างแบบร่างแห 3 มิติ ส่งผลต่อการปรับปรุงคุณสมบัติความยืดหยุ่นของฟิล์มไคโตแซน และเพิ่มคุณสมบัติการชอบน้ำของฟิล์มไคโตแซน

ฟิล์มไคโตแซนคลอสลิงค์กับยางธรรมชาติอิพ็อกซิไดซ์ปราศจากโปรตีนบรรจุยาไดโครฟีแนคสามารถเตรียมได้โดยวิธีการระเหยแห้ง พบว่าฟิล์มไคโตแซนมีประสิทธิภาพการกักเก็บยาที่สูงทุกสูตรการทดลอง และยามีการกระจายตัวที่ดีในแผ่นฟิล์ม ผล FTIR แสดงให้เห็นว่าโมเลกุลของยาไดโครฟีแนคเกิดอันตรกิริยาพันธะไฮโดรเจนกับฟิล์มไคโตแซน ผล XRD และ DSC พบว่ายามีการกระจายตัวในฟิล์มแบบอสัณฐาน การปลดปล่อยยาจากฟิล์มไคโตแซนคลอสลิงค์กับยางธรรมชาติอิพ็อกซิไดซ์ปราศจากโปรตีนทั้ง 2 ชนิด พบว่ามีรูปแบบการปลดปล่อยยาแบบช้า ในทางตรงกันข้ามฟิล์มไคโตแซนที่ไม่ได้คลอสลิงค์และฟิล์มไคโตแซนคลอสลิงค์กับเอธิลีนไกลคอลไดกลัยซิดิลอีเธอร์มีรูปแบบการปลดปล่อยยาที่เร็วกว่า การปลดยาไดโครฟีแนคจากฟิล์มไคโตแซนคลอสลิงค์กับยางธรรมชาติอิพ็

อกซิไดซ์ปราศจากโปรตีนทั้ง 2 ชนิด พบว่ามีการปลดปล่อยยาตามโมเดลของคอสเมเยอร์เปปปาสซึ่งอธิบายว่าอันตรกิริยาระหว่างยากับองค์ประกอบของฟิล์มมีบทบาทสำคัญในการละลายยา ยาสามารถที่จะละลายแพร่ผ่านรูของร่างแหของฟิล์มได้ การศึกษาความคงตัวของยาไดโครฟีแนคในฟิล์มไคโตแซนพบว่าจะต้องเก็บฟิล์มไคโตแซนที่อุณหภูมิต่ำกว่า 40 องศาเซลเซียส และมีการควบคุมความชื้น

ไคโตแซนไมโครสเฟียร์ถูกพัฒนาโดยการทำปฏิกิริยาคลอสลิงค์ของไคโตแซนกับยางธรรมชาติอิพ็อกซิไดซ์ปราศจากโปรตีนร้อยละ 25 และยางธรรมชาติอิพ็อกซิไดซ์ปราศจากโปรตีนร้อยละ 50 ซึ่งบรรจุยาไดโครฟีแนคเพื่อใช้ในระบบนำส่งยา สูตรการเตรียมไคโตแซนไมโครสเฟียร์บรรจุยาได้โครฟีแนคที่เหมาะสม ใช้ 1.5 กรัม ไคโตแซนละลายใน 100 มิลลิลิตรของ 1% สารละลายกรดอะซิติก, 1 มิลลิลิตร ของ 4% ยางธรรมชาติอิพ็อกซิไดซ์ปราศจากโปรตีนร้อยละ 25 หรือ 1 มิลลิลิตร ของ 1% ยางธรรมชาติอิพ็อกซิไดซ์ปราศจากโปรตีนร้อยละ 50 ที่ละลายในเตตระไฮโดรฟิวแรน ไดโครฟีแนค 20 มิลลิกรัม และ 1 มิลลิลิตรของ 1% ไตรโพลีฟอสเฟต การเตรียมไมโครสเฟียร์ทำโดยใช้เทคนิคสเปร์ดราย ตามลำดับ

ขนาดของไมโครสเฟียร์ที่ได้มีขนาดเฉลี่ยอยู่ในช่วง 6-10 ไมโครเมตร เมื่อวัดด้วยเครื่องวัดขนาดอนุภาค และจาการศึกษาด้วยเทคนิคการใช้กล้องจุลทรรศน์อิเลคตรอนพบว่าไมโครสเฟียร์ที่ได้เกิดจากการเกาะกันของทรงกลมขนาดนาโนที่มีขนาดน้อยกว่า 1 ไมโครเมตร ไคโตแซนไมโครสเฟียร์บรรจุยาไดโครฟีแนคที่ได้มีลักษณะเป็นผงสีเหลืองอ่อน ขนาดอนุภาคของไคโตแซนไมโครสเฟียร์ที่ได้อยู่ในช่วง 8.24-10.89 ไมโครเมตร เมื่อวัดด้วยเครื่องวัดขนาดอนุภาค และมีการกระจายขนาดน้อยมาก และศักย์ซีตามีค่า 29.74$\pm$2 และ 38.42$\pm$3.06 มิลลิโวลต์ ตามลำดับ การกักเก็บยาไดโครฟีแนคในไมโครสเฟียร์ทุกสูตรตำรับพบว่ามีประสิทธิภาพสูง

ผล FTIR แสดงว่าโมเลกุลของยาเกิดอันตรกิริยากับไคโตแซน DSC แสดงให้เห็นว่าโมเลกุลของยามีการกระจายตัวเป็นอย่างดีในไคโตแซนไมโครสเฟียร์ และ XRD แสดงว่าไดโครฟีแนคในไมโครสเฟียร์อยู่ในรูปแบบอสันฐาน

การปล่อยยาของไมโครสเฟียร์คลอสลิงค์กับยางธรรมชาติอิพ็อกซิไดซ์ปราศจากโปรตีนทั้ง 2 ชนิด พบว่ามีการปลดปล่อยยาเป็นไปตามรูปแบบของ ฮิกซันและโครเวลล์ การศึกษาความคงตัวของยาพบว่ายาไมโครฟีแนคที่อยู่ในไมโครสเฟียร์คลอสลิงค์กับยางธรรมชาติอิพ็อกซิไดซ์ปราศจากโปรตีนทั้ง 2 ชนิด มีความคงตัวมากกว่าไดโครฟีแนคในไมโครสเฟียร์ที่ไม่ถูกคลอสลิงค์ทุกอุณหภูมิที่ทำการทดลอง การใช้ตัวคลอสลิงค์ร่วมทำให้เกิดไคโตแซนแบบร่างแหสามารถที่นำมาประยุกต์ใช้ทางชีวการแพทย์ในอนาคตได้

ฟิล์มไคโตแซนที่บรรจุไมโครสเฟียร์ที่คลอสลิงค์ด้วยยางธรรมชาติอิพ็อกซิไดซ์ปราศจากโปรตีนที่มียาไดโครฟีแนคเป็นยาต้นแบบพบว่ามีการกักเก็บยาในปริมาณสูง SEM พบว่ามีการกระจายตัวของไมโครสเฟียร์ในฟิล์มอย่างสม่ำเสมอ การปลดปล่อยยาออกมาจากไมโครสเฟียร์เฟียร์คลอสลิงค์กับยางธรรมชาติอิพ็อกซิไดซ์ปราศจากโปรตีนทั้ง 2 ชนิดที่บรรจุในฟิล์มไคโตแซนพบว่ามีการปลดปล่อยยาช้ากว่าไมโครสเฟียร์ที่ไม่ถูกคลอสลิงค์ การปลดปล่อยยาเป็นไปตามรูปแบบการปลดปล่อยของคอสเมเยอร์เปปปาส

Thesis Title Preparation of Chitosan Films and Microspheres by Cross-linking Reaction Using De-proteinated Epoxidized Natural Rubber for Skin Drug Delivery

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ABSTRACT

Chitosan films can be prepared successfully by cross-linking reaction using ENR as a cross-linker. ENR-25 and ENR-50 were utilized in this study. De-proteinated ENR (dP-ENR) were prepared by removing any protein remaining by extraction process and will be used throughout the investigation. In chitosan film preparation, 1.5% w/v of chitosan (MW 300KDa) was used to perform cross-linking reaction with 1 mL of 1% w/v dP-ENR-50 or 4% w/v dP-ENR-25 in THF. The chitosan films were obtained by casting method at 40 ºC. The resulting products were found to have better mechanical property for example, better swelling property, higher moister uptake capacity, increate tensile strength and elongation at break. They also demonstrated to have no skin irritation. The cross-linking reaction should occur via nucleophilic ring opening reaction of epoxide ring of ENR provides three dimension network with improved elasticity and hydrophilicity of the chitosan film.

The films of chitosan cross-linked with dP-ENRs containing diclofenac drug were successfully obtained by casting method. High drug entrapment efficiencies were observed in all film formulations and the drug was well distributed throughout the films. FT-IR results demonstrated that the drug molecule has interaction with chitosan's moieties, especially by hydrogen bonding interaction. The drug was well dispersed within the films in an amorphous form indicated by DSC and XRD results. CH-dP-ENRs films provided sustained release patterns of the drug from the film whereas uncross-linked CH-film and CH-EGDE film gave fast drug release pattern.

Diclofenac was released from CH-dP-ENRs films in Korsemeyer-peppas model which the interaction of the drug and film compositions played an important role in drug solubility property. The dissolved drug diffused from the porous network of the film during solubility study was observed. To preserve drug stability in the chitosan films, the films should be stored in low temperature (< 40 °C) in a controlled humidity.

Chitosan microspheres was developed by cross-linked reaction with dP-ENR-25 or ENR-50 containing diclofenac for drug delivery system. The suitable formulations of chitosan microspheres cross-linked with dP-ENR-25 or ENR-50 containing diclofenac were using 100 mL of 1.5% w/v chitosan (MW 300 KDa) in 1% acetic acid solution, 1 mL of 1% dP-ENR-50 or 1 mL of 4% dP-ENR-25 in THF, diclofenac 20 mg and 1 ml of 1% TPP respectively. The microspheres were obtained by spray drying method.

The sizes of the obtained microspheres were found to be in a range of 6-10 μm by particle analyzer. Microspheres were formed by the aggregation of nanoparticles with their size were less than 1 μm. Chitosan microspheres cross-linked with dP-ENRs containing diclofenac were obtained as light yellow powder. The particle size of chitosan microsphere formulations were obtained in the range of 8.24-10.89 μm (by particle size analyzer) with low PI values and their zeta potential values were 29.74±2-38.42±3.06 mV, respectively. High entrapment efficiencies of diclofenac in microspheres formulations (> 90%) was also achieved.

FT-IR results demonstrated that the drug molecule has interaction with chitosan's moieties. DSC showed that the drug was molecularly dispersed inside the matrix of chitosan microspheres and XRD showed diclofenac in the microspheres was in an amorphous form.

The entrapped drug were slowly release from the microspheres both using dP-ENR-25 and dP-ENR-50 according to the Hixon and Crowell model. Diclofenac entrapped in both chitosan microspheres cross-linked with dP-ENR-25 and chitosan microspheres cross-linked with ENR-50 showed more stable than the diclofenac in chitosan microspheres uncross-linked, when stored in all temperatures. These co-cross linked chitosan networks may be suitable for future biomedical applications.

Chitosan film containing microspheres cross-linked with dP-ENRs having diclofenac as a model drug were prepared. High drug entrapment efficiency was observed. SEM micrograph showed well distribution of microspheres in the film matrix. The drug release from the film loading microspheres cross-linked with dP-ENRs containing diclofenac was found to be much slower than the film containing uncross-link microspheres. The release pattern was according to Korsemeyer-peppas model.

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LIST OF ABBREVIATION AND SYMBOLS

σ	=	the standard deviation of the response
>	=	greater than
<	=	less than
%	=	percentage
θ	=	theta
ABR	=	acrylonitrile butadiene rubber
AFM	=	atomic force microscopy
β-	=	beta-
BSA	=	bovine serum albumin
°C	=	degree Celsius
$CDCl_3$	=	chloroform-d
CH	=	chitosan
CH-dP-ENR-25	=	chitosan cross-linked with deproteinated ENR-25
CH-dP-ENR-50	=	chitosan cross-linked with deproteinated ENR-50
CH-EGDE	=	chitosan cross-linked with ethylene glycol diglycidyl ether
cm	=	centimeter
cm^2	=	square centimeter
CSO	=	chitosan oligosaccharide
DDA	=	degree of de-acetylation
dP-ENR	=	deproteinated epoxidized natural rubber
DSC	=	Differential Scanning Calorimeter
EAB	=	elongation at break
EGDE	=	ethylene glycol diglycidyl ether
ENR	=	epoxidized natural rubber
ENR-10	=	10-mole % of epoxide incorporated into the natural rubber chain

ENR-25	=	25-mole % of epoxide incorporated into the natural rubber chain
ENR-45	=	45-mole % of epoxide incorporated into the natural rubber chain
ENR-50	=	50-mole % of epoxide incorporated into the natural rubber chain
FT-IR	=	fourier transform infrared spectroscopy
g	=	gram
GI	=	gastrointestinal
GPC	=	gel permeation chromatography
^{1}H NMR	=	proton nuclear magnetic resonance
HPLC	=	High performance liquid chromatography
h	=	hours
$I_{2.70}$	=	integrated area of peaks at chemical shifts 2.70
$I_{5.14}$	=	integrated area of peaks at chemical shifts 5.14
Inch	=	inches
IV administration	=	intravenous administration
L/kg	=	liter per kilogram
LB	=	Langmuir-Blodgett
m^2	=	square meter
mg	=	milligram
µm	=	micrometer
min	=	minute
mL	=	milliliter
M_p	=	weigh of rubber (g)
M_w	=	molecular weight
MPa	=	mega pascal
mm	=	millimeter
MMA	=	methyl methacrylate
nm	=	nanometer
ND	=	No detection

NMR	=	Nuclear Magnetic Resonance
NSAID	=	non-steroidal anti-inflammatory drug
Pa	=	pascal
PEG-200	=	polyethylene glycol-200
o/w	=	oil in water
pH	=	potential of hydrogen ion
PI	=	polydispersity index
r^2	=	regression coefficient
RH	=	relative humidity
RT	=	room temperature
rpm	=	round per minute
%RSD	=	percentage relative standard deviation
S.D.	=	standard deviation
SEM	=	scanning electron microscope
Tmax	=	Time at maximum concentration
TS	=	Tensile strength
µg/mL	=	microgram per milliliter
µg/g	=	microgram per gram
UV-vis	=	UV-visible spectrophotometer
λ	=	wavelength
w/w	=	weight by weight
w/v	=	weight by volume
XRD	=	X-ray diffraction

CHAPTER 1

GENERAL INTRODUCTION

1.1 Skin drug delivery

Skin is one of a widely used route for delivery of both local and systemic drugs. Skin delivery pathway is known as “topical delivery”, which can be designed to deliver drugs across a patient’s skin. Furthermore it may be of interest for cosmetic and cosmeceuticals products development (Prow *et al.*, 2011). Several topical delivery formulations containing drugs such as ointments (Wu *et al.*, 2013), creams (Krishnaiah *et al.*, 2014), lotions (Czerwinski *et al.*, 2014), liniments (Wang *et al.*, 2010), gels (Das *et al.*, 2013), pastes (Kakigi *et al.*, 2009), powders (Chen *et al.*, 2014), microspheres (Fu *et al.*, 2009) and films (Mohanraj *et al.*, 2014) are widely used in many products development. These delivery systems have different advantages and disadvantages depend on types and sites of applications.

Transdermal Drug Delivery (TDD) has possibility for controlling drug delivery through skin (Zan *et al.*, 2005). Skin’s barrier function is accomplished, by outermost of skin’s few microns such as stratum corneum, compositionally and morphologically biomembrane. This is very thin [approximately 0.4–10 μm], least permeable of skin layers provide limitation in process epidermal differentiation by forming of compressed keratin filled corneocytes anchored in laminate lipophilic matrix. These extracellular matrix lipids provide several functions involving in skin delivery system as following. (1) They give diffusion pathway and continuous phase from surface of skin to base of stratum corneum (sc). (2) Compositions including cholesterol, free fatty acids, and ceramides, are unique biomembranes. Absence of phospholipid is particularly noteworthy. (3) Despite deficit of polar bilayer forming lipids, sc lipids act as sheets of

multilamellar. (4) Predominantly saturated, long chain hydrocarbon tails make interdigitated configuration high, and ordered. Gel phase membrane domain formation is liquid crystalline membrane systems. Moreover, transdermal mode offers several advantages in drug delivery system since skin shows large accessible and surface area of 1–2 m^2 available for drug absorption. A patch-like device application to skin surface is a noninvasive procedure, allowing continuous intervention such as system removal, repositioning or replacement, therefore promote patients compliant. Further benefits of transdermal drug delivery systems are emerged in last few years. These include sustained release possibility which is useful to short biological half-live drugs which may require parenteral administration, frequent oral administrations. The narrow therapeutic drugs are also suitable for this type of delivery system. (Naik *et al.*, 2000).

1.1.1 Human skin

It is known that the body largest organ is skin, weighing more than 10 % of body mass in total. Forming of human body‘s outermost layer, provides two main functions. They are protection and communication. (Brown *et al.*, 2006). Communicative function depends on biochemical transmitted signals, pigmentation and neuroreceptors. Protective function protects penetration to the body. It also prevents loss of substances from body. From these aspects, skin can protect body from physical environment (radiation, abrasion), chemical factors (toxic substances) or biological (microorganisms). However, body’s ion loss and water loss are protected. In addition, skin includs sweat glands, circulation system and hair follicles. They enable thermos-regulation to make sure the right function of biochemical apparatus. Regional variations in morphology of skin have occurred despite of general role. Stratum corneum’s compositions and thickness also varies. Appendages presence and hair follicles over body surface is not constant. These variations are originally functions. They give soles protection against any aggressive abrasion. Skin’s basic structure is shown in Figure 1. Two layers of skin are epidermis layer and dermis layer. They are adjacent subcutaneous to fat tissue. Body outer layer is formed by epidermis. They

comprise of non-viable in viable cells and stratum corneum layers. Epidermal dermal junction's basal cell layer generates epidermis. Keratinocytes differentiate and undergo composition changes and also changes in structure on their way to skin surface. They are done during migration through stratum granulosum and stratun spinosum. Final differentiation occurs in stratum corneum. Substances' major barrier to permeate across epidermis is formed by stratum corneum. Stratum corneum consists of dead keratinocytes. They are coated from impermeable proteinous cornfield envelope (Prow *et al.*, 2011). Lipid matrix is surrounded by corneocytes and exists is in many layers in lipid lamellae. Intercellular lipid matrix forms mainly rather tortuous pathway for substances across stratum corneum permeation. It is due to impermeable cornfield envelope. Dermis locates beneath epidermis. Thin undulating epidermis is indirect contact with upper dermis. It is papillary dermis. In addition, dermis' main thicker part is reticular. It is a fibrous, amorphous connective tissue and filamentous. They consist of collagen, elastin, fibroblasts and ground substance. Their main role is to provide structure of epidermis and embedded. They support blood vessels, sebaceous glands, hair follicles, nerves, sweat, and skin's elasticity. Tissue is highly vascularized, contrasting to epidermis. Dermis is underlying by subcutaneous fat tissue. It is fat cell assembly. They are linked by collagen fibers which create energy storage, body's mechanical cushion and thermal barrier (Narasimha and Shivakumar, 2010).

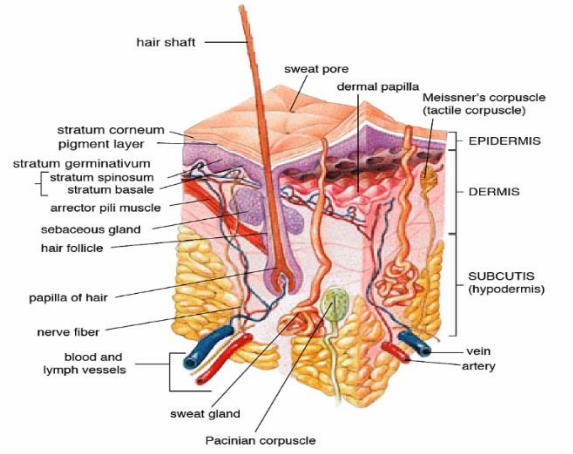


Figure 1. Schematic crosssection of human skin (From Shah *et al*., 2011).

1.1.2 Drug penetration pathways through skin

When apply drugs on surface of skin, drug will penetrate into, and through skin via different routes. Drugs can be penetrated through stratum corneum (trans epidermal) or through appendages (trans-appendageal) (Figure 2). Two possible routes can be identified during penetration via stratum corneum. 1) Penetration alternating via lipid lamellae and corneocytes (transcellular route). 2) Penetrating through tortuous pathway or intercellular route (lipid lamellae). Penetration's predominant route via stratum corneum is the intercellular route. It is caused by densely mainly crosslinked cornfield envelope keratinocytes' coating. Small size hydrophilic molecules' cannot be transported easily through this pathway. Route of appendageal or shunt route includes sweat gland duct or follicular duct. Sweat glands' main content is hydrophilic. Content of follicular duct is lipophilic. This is mainly for sebum excreted to the opening of follicular duct (Benson, 2005, Ylva and Joke, 2005, Shah *et al.*, 2011).

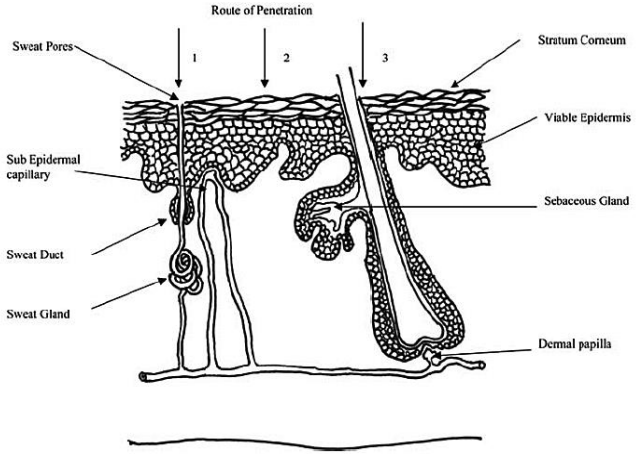


Figure 2 Skin penetration routes 1. through sweat ducts; 2. via hair follicles; 3. directly across stratum corneum; (From Benson, 2005).

1.1.3 Factors influencing extent of the absorption of drug and topical products through skin (Sandhu *et al.*, 2012)

1.1.3.1 Skin microflora

A microbial population which is supported by skin surface has possibility to biotransformations of therapeutic agents. However, there are little information to suggest that microbial can transform compounds that applied on skin for percutaneous absorption. They have significance effect than action of metabolism action of the skin.

1.1.3.2 Skin pH

It is known that pH of topical formulation could affect extent of ionizable drug molecules dissociation and thermodynamic activity. The surface of human skin normally has pH about 4.6 (Hanson *et al.*, 2002). There, it many give an effect to the partitioning and penetration of drug through skin.

1.1.3.3 Lipids of Skin Surface

Skin that possesses sebaceous glands secretes a mixture of lipids which can form irregular thin film on skin surface having the thickness about of 0.4 to 4 μm.

1.1.3.4 Temperature

Change of temperature on or into skin is often accompanied by body physiological reactions, for example increase in blood flow or horny layer's moisture content increase could be observed when the body temperature has changed. These factors can provide higher percutaneous drug absorption. Nevertheless, temperature increase could also increase solubility of drug in formulation vehicle, diffusivity, and stratum corneum. These effect could enhance and improve an increase in percutaneous absorption.

1.1.3.5 The Flow of Blood

Transepidermal absorption process to cutaneous can be effected by microcirculation which could bring compounds to circulation system or to underlying tissues. The levels of concentration and substances accumulation in skin's dermis deeper parts can be modified by cutaneous blood flow. Vasoactive drugs which are

applied topically and systemic could also give effect to drug preparation. Decreased of blood flow can be obtained through ligation. It can modulate drugs' transdermal delivery.

1.1.3.6 Effect of skin metabolism

Metabolic activities including oxidative, hydrolytic, reductive, and conjugative reaction make skin as an extrahepatic metabolism source of various xenobiotic. It also can applied drugs topically. Skin's diffusional and metabolic process is much related with one most likely to have a profound effect on other.

1.1.3.7 Effect of age

Diminished surface lipid compositions and concentrations of old skin reduced dissolution medium for topically administered compounds. It is reasonable to speculate this physiological change. It will therefore affects solubility property of lipid soluble compounds. Therefore, pharmacodynamics parameters which suggest penetration or reducing effect have to be carefully used. In contrast, permeability of the skin is greater in premature infants.

1.2 Topical delivery dosage forms

These are a number of dosage forms have been designed to be applied on skin including ointments, lotions, creams liniments, gels, pastes, films and powders. Various applied dosage forms can be defined below (Prabhjotkaur and Khan, 2013).

Ointments and can be medicated or no medicated. They are semisolid preparations for external use. They have lubricating effect or emollient after skin application (Prabhjotkaur and Khan, 2013).

Creams are a semisolid emulsion formulated for application to skin or mucous membranes. Diameter of droplets in topical emulsions are generally in a ranges of 0.1–100 μm. Cream emulsions are the most commonly obtained in oil in water formulation but may also be found as water in oil. Former readily rub to the skin

(hence the term “vanishing” cream) and are removed either by licking or washing. In contrast to the latter are cleansing and emollient. Water in oil emulsions can spread more easily than ointments. They are normally less greasy than ointment. They also can be used in the inflamed skin as the water consequence in the evaporating formulation (Prabhjotkaur and Khan, 2013).

Pastes are different from ointments since they contain high solid material. They are for external application only. They are stiffer and thicker than ointment (Prabhjotkaur and Khan, 2013).

Lotions are also intended for external application. They are categorized as liquid preparations. They often contain powdered substances. They are normally insoluble in the medium of dispersion. They are usually prepared by using suspending agents. Lotions are suitable for skin application and are normally found in cosmeceutical products. Their constituents can be used for therapeutic or protective mean (Prabhjotkaur and Khan, 2013).

Gels/jellies are semisolids systems. Liquid phase is distributed within a 3-dimension polymer matrix and have a higher degree in chemical or physical crosslinking. It consists of gum which can be synthetic or natural.

Gels are aqueous colloidal suspensions of soluble medicament’s hydrated form, and are richer in liquid than magma.

Jellies are either translucent/transparent non-greasy semisolid gel. It can be transparent as an aesthetically pleasing state. They can be obtained in turbid form according to the polymers used in the formulation. It is present in colloidal aggregates which can make the light disperse (Gupta and Garg, 2002).

Plasters are the forms of semisolid dosage. They contain drug substances incorporated in a large proportion of dispersed solid base. They are ideal for skin’s external application. They are quite thick and can be stiff.

Liniments are also medicinal substance emulsions. They are solutions for alcoholic or oleaginous. They are ideal for external rubbing application. Hydro alcoholic/alcoholic liniments are useful as counterirritants and rubefacients. Oleaginous liniments are primarily employed in massage. They have skin's low irritation than those alcoholic liniments.

Transdermal patch or films

Transdermal delivery patch can deliver therapeutically active agents to circulation system via skin. Transdermal patches have several advantages over oral dosage forms such as can overcoming first-pass metabolism, improve patient compliance, and reduce gastrointestinal side effects (Tanner and Marks, 2008, Guy, 2010). Moreover, transdermal patches offer several advantages such as maintenance of constant and prolonged drug level, reduce frequency of dosage administration, product can be self-administration and ease to remove leading to patients compliance (Keith, 1983, Bertocchi *et al.*, 2005). Main components in transdermal patches or films formulation are normally composed of 1) polymer matrix 2) drug 3) permeation enhancers and 4) adhesive. Polymer matrix is a backbone of delivery system which important for control of drug. Polymers that will be used in transdermal patch or film preparations should be chemically nonreactive, stable, nontoxic and cheap. A number of polymer matrixes have been reported to be used in transdermal patches or films preparation for example cellulose derivatives, zein, gelatin, synthetic polymers and rubber such as polyvinyl alcohol (PVA), polyethylene (PE), polyamide, polyacrylate, etc. Chitosan is one of widely used for investigation possibility utilization in transdermal patches or films formation since it has property in accordance with previous described (Ghosh *et al.*, 2010). Several methods have been utilized in chitosan film formulations for drug delivery such as interaction with anions for example, tetrapolyphosphates, alginate, octyl sulphate, tripolyphosphates, lauryl sulphate, thermal crosslinking, solvent evaporation, spray drying, chemical crosslinking and etc. For instance, chitosan films crosslinked by tripolyphosphate exhibited better pH sensitive swelling and controlled release properties of riboflavin for oral delivery (Shu and Zhu, 2002). Film of chitosan and polyethylene glycol was constructed for

controlled release ciprofloxacin hydrochloride for skin drug delivery (Wang *et al.*, 2007). Chitosan films prepared by crosslinking with ethylene glycol diglycidyl ether gave films having a good conformity with microstructure of film (Ghosh *et al.*, 2010). Use of microspheres and nanoparticles in dermatopharmaceutical applications as well as in cosmetics has found to be of interest to many product development researchers. For drug delivery, chitosan nanospheres and microspheres have been formulated. For instance, Mi *et al* (1999) used ethylene glycol diglycidyl ether crosslinked with chitosan solution. They form high porous chitosan microspheres (Mi *et al.*, 1999). Chitosan has been extensively used in various topical delivery systems, according to good bioadhesive property and sustains active constituent ability and release. Bioadhesive chitosan microspheres for cetyl pyridinium chloride's topical sustained release have evaluated. Microbiological activity was improved by micro particulate systems. Conti et al (2000) prepared microparticles. It consists of chitosan, and designed it for topical wound healing. Blank and ampicillin loaded microspheres preparation were doned by spray drying technique and evaluated in albino rats showed that both drug loaded and blank microspheres demonstrated good wound healing properties (Conti *et al.*, 2000). Varshosaz *et al* (2006) prepared gel containing lidocaine as a local anesthetic agent with variation in molecular weights, also with concentrations of chitosan for prolonging anesthetic effect transdermal delivery. In this study, lecithin was used as an enhancer of permeation. Viscosity, drug release from synthetic membranes, bioadhesion, and drug permeation via rat skin or biological barrier was studied. It was illustrated that by increasing concentrations and molecular weights of chitosan, increasing in both rate and extent of drug release were observed that could be probably because of repulsive forces increase between lidocaine and chitosan cations (Varshosaz *et al.*, 2006). Chitosan microparticles consisting with minoxidil sulphate were developed. They were prepared by spray drying technique in order to study ability for it's sustain drug release and further utilized in the delivery system target. It is suitable for topical treatment of alopecia. Around 82% of high encapsulation efficiency is observed by microparticles. It's shown a mean diameter of 3.0 μm, and a spherical morphology without porosities. Chitosan microparticles underwent instantaneous swelling, when suspended in an ethanol or water solution. It was increasing 90% of mean diameter after soaking.

Chitosan microparticles were able to sustain the release of many active compounds (Gelfuso *et al.*, 2011). Vitamin E incorporated chitosan microspheres for topical were prepared using spray drying method to improve stability of vitamin E for cosmetic propose. Results justified that vitamin E was successfully incorporated into microsphere of chitosan. Due to the studies of *in vitro*, chitosan microspheres had shown burst release during first 5 minute after application. However, release lasted for 6 h. *In vivo* studies showed that chitosan microspheres contains vitamin E is a promising formulation for an antiaging effect. It is to decrease in skin roughness and to increase in the skin moisture and elasticity (Yenilmez *et al.*, 2011). Insulin is normally used for the treatment of patients with type I diabetes, however, three or four injections are needed daily for best control of diabetes which definitely affect patient compliance (Elsayed *et al.*, 2014). Chitosan microspheres containing insulin have been developed for topical delivery. The system can release levels of therapeutic of bioactive insulin for many weeks. It has the possibility to sustain and stimulate the treatment. It can be done by encapsulating the crystalline insulin within poly(*D*,*L*-lactidecoglycolide) microspheres (Hrynyk *et al.*, 2010).

1.3 Advantages and disadvantages of topical drug delivery (TDD) (Gaikwad, 2013)

Topical drug delivery system has shown to have both advantages and disadvantages which are summarized below

1.3.1 Advantages

Since skin presents readily accessible and relatively large surface area (1–2 m^2), hence, drug absorption can be enhanced compared to other routes.

TDD can avoid gastrointestinal drug absorption difficulties caused by gastrointestinal pH, enzymatic activity, drug interactions with food, drink, and other orally administered drugs.

TDD can be substituted for oral administration of medicines when that route is unsuitable for patient's treatment, as with vomiting and diarrhea.

TDD can avoid first pass metabolism of drug. It is initial pass of a drug substance through portal and systemic circulation, following gastrointestinal absorption (thereby possibly avoiding deactivation of drug by digestive and liver enzymes).

Topical drug delivery was convenient for parenteral therapy. Drug therapy could be terminated rapidly by removing its application from skin surface. If toxicity develops from a drug administered topical, effects could be moderated by removing from skin. Topical delivery can be used as an alternative delivery system for patients who cannot tolerate oral dosage forms.

1.3.2 Disadvantages

The most suitable candidate drugs for topical drug delivery pathway are relatively potent drugs only. Since drug entry's natural limits imposed by impermeability of the skin. Some patients have developed contact dermatitis at application site from at least one of system components, resulting in discontinuation. For drugs that required high blood levels, this delivery system cannot use as well. Finally, topical delivery usage can be more expensive than other application routes.

1.4 Polymers used in film and microspheres preparation

In recent years, biomedical materials for drug delivery system have been of interest to many researchers. Polymers are good candidates which can be used to control release of drugs from device. In order to a polymer to be used in transdermal system, below criteria should be satisfied. Likely to be useful polymers for transdermal devices are; summarized in Table 1. One of materials often used in biomaterial production is chitosan. Chitosan is a polysaccharide. Ii is obtained by deacetylation of alkaline of naturally occurring abundant polysaccharide, chitin (Mi *et al.*, 2003, Zhao *et al.*, 2009). Chitin is a principal component of crustaceans's protective cuticles.

For example crabs, shrimps, lobsters, prawns, and cell walls of some fungi such as mucor and aspergillus. Chitin is a straight homopolymer. It composed of β-(1,4)-linked-*N*-acetylglucosamine units. While chitosan comprises of those copolymers' glucosamine and *N*-acetylglucosamine (Chawla and Singla, 2001, Kato *et al.*, 2003). Structure of chitosan is displayed in Figure 3.

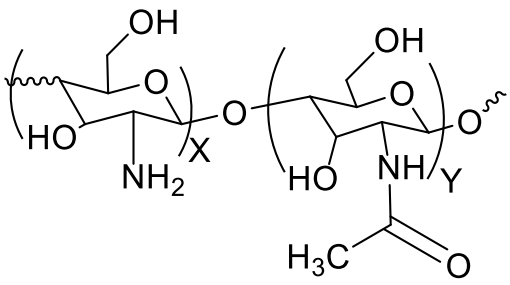


Figure 3. Structure of chitosan (Ravi and Majeti, 2000).

Chitosan contains many free amino groups having positive charge. They are readily to react with polymers or many negatively charged surfaces (Sinha *et al.*, 2004). It is a weak base. It is also insoluble in water and organic solvents. However, it is soluble in dilute aqueous acidic solution (pH < 6.5). It can convert glucosamine units into a soluble form, $R–NH_3^+$. It can get precipitated in alkaline solution or with polyanions. It also can forms gel at lower pH. Chitosan is commercially available in dry flakes, fine powder form and solution. It has between 3,800 and 2,000,000 of an average molecular weight. It has %deacetylation in a range of 66 to 95%. Physical properties of chitosan including particle size, viscosity, density, molecular weight are important chitosan characteristics, degree of deacetylation. They influence the properties of pharmaceutical formulations on chitosan (Sinha *et al.*, 2004). There are several advantages of chitosan such as good biocompatibility, low toxicity, biodegradability, antibacterial, non-immunogenic, non-carcinogenic, and wound healing for using in

pharmaceutical formulations. Pharmaceutical formulation including powders, films, beads (Guo et al., 2005, Chen *et al.*, 2006, Igberase *et al.*, 2014), hydrogels (Mukhopadhyay *et al.*, 2014, Zang *et al.*, 2014), tubular (Wang *et al.*, 2005, Chen and Syu, 2012), conduits (Liao *et al.*, 2013), porous scaffolds (Gorczyca *et al.*, 2014), tissue engineering and drug delivery vehicle (Kuo *et al.*, 2014) were performed. Currently, chitosan has been examined. It was in the industry of pharmaceutical *extensively* for its possibility usage of drug delivery system development.

Table 1 Polymers utilized in films and microspheres formulations.

Polymer	Type of polymer	Formulation	Sources
Cellulose derivatives	Natural polymers	Films Microspheres	Eronen *et al*., 2011, Zhang *et al*., 2012, Yuan *et al*., 2015
Natural rubber	Natural polymers	Films Microspheres	Simchareon *et al*., 2012, Soheilmoghaddam *et al*., 2013
Starch	Natural polymers	Films Microspheres	Lin *et al* 2013 Kim *et al* 2014
Polyacrylate	Synthetic polymers	Films	Huang *et al* 2014
Polyvinylpyrrolidone	Synthetic polymers	Films	Wang *et al* 2014
Polymethyl methacrylate	Synthetic polymers	Films	Chu *et al* 2012
Hydroxypropylmethyl cellulose	Synthetic polymers	Films Microspheres	Nokhodchi *et al*., 2002, Ghosh *et al*., 2014
Carboxymethyl cellulose	Synthetic polymers	Films	Dashipour *et al*., 2015

1.5 Chemical crosslikers

Up to now, drug delivery formulations that based on chitosan (such as beads, films, microspheres, etc.) were usually prepared by chemical crosslinking reaction (Shu and Zhu, 2002). In chemical crosslinking reaction irreversible covalently link bonds were formed in covalently crosslinked chitosan. Crosslinking reaction is influenced mainly by size and type of crosslinking agent and chitosan's functional groups. In crosslinked chitosans, polymeric chains are interconnected. Those are done by crosslinker, leading to formation of 3-dimention network (Gonçalves *et al.*, 2005). Therefore, chitosan micro or nano structure and physical properties, including mechanical, chemical, and thermal, and biological stabilities were modified. Certain chemicals such as genipin, glutaraldehyde, ethylene glycol diglycidyl ether and etc. are applied commonly to modulate physico-chemical properties of chitosan (Ghosh *et al.*, 2010).

1.5.1 Crosslinking agent classification

Crosslinking agents are classified on basis of crosslinking interaction with chitosan during crosslinking reaction. These are physical and chemical crosslinking.

1.5.1.1. Physical crosslinking mechanism has increased interest in recent years. Main reason is to avoid the use of chemical crosslinking agent which may be toxic. For example, polysaccharides are formed crosslinking network with counter ions at surface. Those high counter ion concentration and longer exposure times would require to achieve completion of crosslinking of polysaccharides. Physical crosslinking could occur by thermal crosslinking using citric acid, phosphoric acids, or dextran sulfate.

1.5.1.2. Chemical crosslinking is a highly versatile method which can provide the cross-linked product with a good mechanical stability. This type of crosslinking, covalent bonds is present between polymer or polymer chains and

crosslinker. During crosslinking reaction, cross-linking agent diffuse to polymeric and react with polysaccharides, either form intramolecular or intermolecular linkages. Factors that affect chemical crosslinking are concentration of crosslinking agent and cross-linking time. Chemical crosslinking of chitosan can be carried out by using glutaraldehyde, formaldehyde, genipin, vanillin, radical polymerization, 1,6-hexamethylene diisocyanate or 1,6-hexane dibromide and ethylene glycol diglycidyl ether (RemuñánLópez and Bodmeier, 1997, Aggarwal and Pahuja, 2013).

1.5.2 Properties, Characteristics, and advantages of crosslinking materials (Bailey *et al.*, 1999, Arrascue *et al.*, 2003, Aggarwal and Pahuja, 2013)

Crosslinked materials are homogeneous and not difficult to prepare with relatively inexpensive reagents. They are available in many types of characteristic with various properties, and in numerous configurations such as gels, particles of irregular shape or regular beads, coatings, membranes, films, fibers, sponges and capsules.

Crosslinked gels are much stable hydrophilic polymers. They are normally insoluble in alkaline and acidic mediums as well as in a number of organic solvents. They are more resistant to shear, high temperature and low pH, comparing to their parent polysaccharides.

Chitosan beads formed by crosslinking reaction could maintain properties of their original characteristics except crystallinity and strength in acidic and basic solutions. These characteristics are important for adsorbant capacity because they can be used in a lower pH environment. Nevertheless, swelling behavior of beads in water is optimized.

Cross-linking reaction can reduce amount of crystalline domains in the polysaccharide. It can change crystalline nature of raw polymer. This parameter influences sorption properties of the product significantly because it may control access to the sorption sites.

In addition, cross-linked beads have other advantages such as increased ease of operation, faster kinetics, and interesting diffusion properties. Due to hydrophilic nature of crosslinking units, these materials are normally have remarkably high swelling capacity in water, and consequently their networks are expanded sufficiently to allow a fast diffusion process for adsorbents. Normally, cross-linked polysaccharides have good sorption properties, however, their adsorption capacities can be justified by grafting of various functional groups onto polymer backbone or polymer network. This could increase sorption site density. Presence of new functional groups on bead surface is results in an increase of surface hydrophobicity and polarity. It could enhance sorption of polar compounds and improves sorption selectivity for target adsorbents.

1.5.3 Crosslinking Mechanism (RossMurphy, 1994)

Crosslinking reaction is influenced mainly by size and type of crosslinkers and functional groups of chitosan. Smaller molecular size of crosslinker, could provide faster crosslinking reaction as it could diffuse easier which depending on crosslinker nature. Main interactions forming network are ionic or covalent bonds. Degree of crosslinking reaction in covalently crosslinked hydrogels may affect their main parameters that influence important properties. For example mechanical strength, swelling, and drug release. They generally exhibit pH sensitive drug release and swelling by diffusion through porous structure. Therefore, hydrogels based on ionically and covalently crosslinked chitosan can be considered as good candidates for drugs oral delivery. Concerning over chitosan microparticle disintegration, it led to their modification by crosslinking to make more rigid polymer to use as a core material in research of controlled drug release. Ko *et al* (2002) reported that use of complexion between polyanion tripolyphosphate and cationic chitosan by electrostatic forces, resulting in more homogeneous structure due to more homogeneous crosslinking process. Crosslinking reaction mechanism of chitosan with different crosslinkers has described below:

1.5.3.1 Genipin

Genipin is an aglycone compound obtained from *Gardenia jasminoides*. Genipin (Figure 4) is an excellent natural crosslinker for collagen, proteins, gelatin, and chitosan. It has been widely used due to its low toxicity. Those crosslinking reaction mechanisms of chitosan with genipin (Figure 5) start from amino groups of chitosan via a nucleophilic attack. It is done by amino group on olefinic carbon atom at C3 of deoxyloganin aglycone, following by opening of dihydropyran ring and is attacked by secondary amine group on the resulting aldehyde group. Novel chitosan gel beads were synthesized by genipin, a coupled ionic and chemical co-crosslinking with tripolyphosphate and naturally occurring crosslinking reagent. Result revealed that ionic or chemical co-crosslinked chitosan networks could be suitable for biomedical application (Mi *et al.*, 2003).

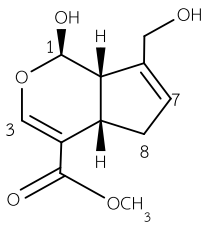


Figure 4. Structure of genipin (Muzzarelli, 2009).

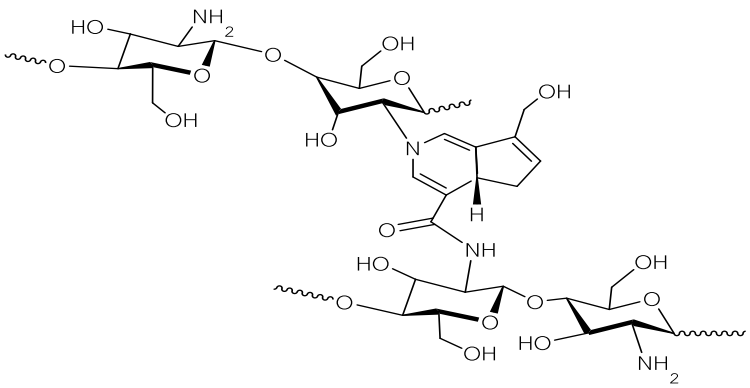


Figure 5. Structure of chitosan crosslinked with with genipin (Muzzarelli, 2009).

1.5.3.2 Glutaraldehyde

Chitosan can be crosslinked easily by dialdehydes. Glutaraldehyde (1, 5-pentanedial, Figure 6) is extensively used in protein immobilization by crosslinking reaction through amino groups. Free pendant amine groups of chitosan polymer can be randomly interact with both aldehyde groups of glutaraldehyde to form stable crosslinked imine bonds (Beppu *et al.*, 2007) (Figure 7).

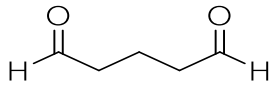


Figure 6. Structure of glutaraldehyde (Gonçalves *et al.*, 2005).

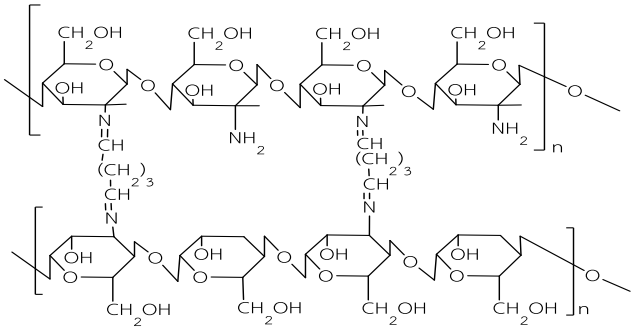


Figure 7. Structure of chitosan crosslinked with glutaraldehyde (Gonçalves *et al.*, 2005).

There are a number of publications using glutaraldehyde as a crosslinking reagent in preparation of chitosan microspheres, for example; microspheres of chitosan were prepared by crosslinking reaction between chitosan with glutaraldehyde and glyoxal and used for sustained release of centchroman (Gupta and Jabrail, 2007). Controlled release of chitosan microspheres containing diclofenac sodium were prepared by coacervation method and crosslinking reaction using glutaraldehyde (Gonçalves *et al.*, 2005). Moreover ketotifen loaded microspheres of chitosan were prepared by crosslinked with glutaraldehyde, and spraydrying method for controlled release property (Guerrero *et al.*, 2010).

1.5.3.3 Epichlorohydrin

Epichlorohydrin (1-chloro-2,3-epoxypropane Figure 8) is a volatile, organic compound of epoxide family. Epichlorohydrin is used mainly in manufacture of glycerin, phenoxy and epoxy resins, to a lesser extent, in manufacture of elastomers, surfactants, plasticizers, ion exchange resins, dyestuffs, pharmaceutical products, lubricants adhesives, oil emulsifiers, and water treatment resins (Hutler *et al.*, 2013). Epichlorohydrin is a crosslinking monofunctional agent. It is used for forming covalent bonds with carbon atoms of chitosan's hydroxyl groups, resulting in rupturing of epoxide ring and a chlorine atom removal (Figure 9) (Laus *et al.*, 2010, Tirtom *et al.*, 2012). Wan Ngah *et al* (2008) used epichlorohydrin in crosslinking reaction with chitosan for chitosan beads preparation. The chitosan beads showed low value of surface area and low porosity. Moreover, chitosan microparticles and chitosan beads prepared from chitosan crosslinked with epichlorohydrin can be used for heavy metals adsorption such as Cu(II), Ni(II), Zn(II), and Pb(II) ion in wastewaters were reported (Coelho *et al.*, 2007, Chen *et al.*, 2008, Laus *et al.*, 2010, Chen *et al.*, 2011, Laus and de Fávere, 2011, Tirtom *et al.*, 2012, Shinde *et al.*, 2013).

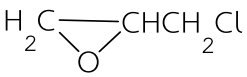


Figure 8. Structure of epichlorohydrin (Laus *et al.*, 2010).

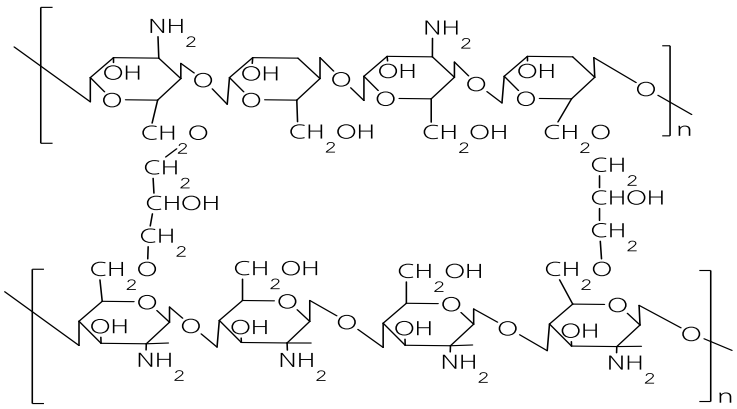


Figure 9. Structure of chitosan crosslinked with epichlorohydrin (Laus *et al.*, 2010).

1.5.3.4 EDGE or Ethylene Glycol Diglycidyl Ether

EDGE or Ethylene glycol diglycidyl ether (Figure 10) is crosslinker for carboxyl, amine and hydroxyl functional groups in polymers preparation. In chitosan's crosslinking reaction with ethylene glycol diglycidyl ether, amine groups of chitosan were nuecleophiles, react by attacking at less substituted carbon of epoxide ring, make ring opening and resulting in chitosan crosslinked polymer (Ngah and Fatinathan, 2008) (Figure 11).

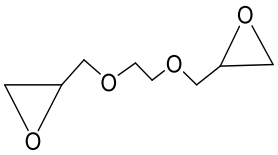


Figure 10. Structure of ethylene glycol diglycidyl ether (Ngah and Fatinathan, 2008).

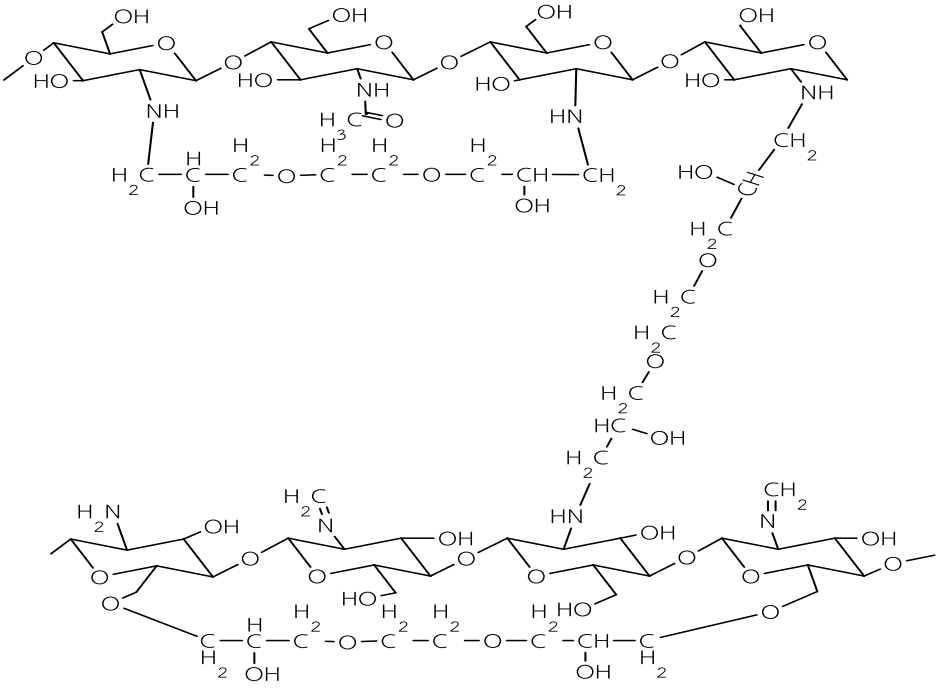


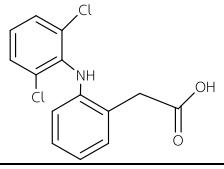
Figure 11. Structure of chitosan crosslinked with ethylene glycol diglycidyl ether (Ngah and Fatinathan, 2008).

Controlled pore size and good mechanical properties on macroporous chitosan membranes could be prepared by crosslinking reaction of chitosan with ethylene glycol diglycidyl ether and used for separations of protein in ion exchange mode (Zeng and Ruckenstein, 1998). Those chitosan beads crosslinked with ethylene glycol diglycidyl ether could increase its chemical resistance and mechanical strength in acid dyes sorption property (Azlan *et al.*, 2009). Paclitaxel loaded chitosan oligosaccharide (CSO) nanoparticles were developed by interfacial polyaddition between amino group of chitosan and epoxy group of ethylene glycol diglycidyl ether in O/W emulsion system (Du *et al.*, 2010). Keratin film was prepared by mixing keratin solution with chemical crosslinkers, ethylene glycol diglycidyl ether, and glycerol diglycidyl ether (GDE) and then the films were obtained by casting method. Chitosan film prepared by chemical crosslinked together with ethylene glycol diglycidyl ether could provide the films that their tensile modulus increase and decreased ultimate elongation without affecting maximum stress (Ghosh *et al.*, 2010).

1.6 Diclofenac

Diclofenac 2-[2-(2,6-dichloroanilino)phenyl]acetic acid, a phenylacetic acid derivative that is a nonsteroidal anti-inflammatory drug (NSAID; Table 2). Like most NSAIDs, diclofenac possesses analgesic, anti-inflammatory, and antipyretic properties. Sodium salt of diclofenac was used to treat osteoarthritis, ankylosing spondylitis, rheumatoid arthritis, and mild to moderate pain. Diclofenac has been most commonly used analgesic in world and is commercially available in various formulations including ones for topical administration (Lenik, 2014, Chae *et al.*, 2015).

Table 2 Diclofenac Properties (From Lenik, 2014)

Systematic (IUPAC) Name	2-[2-(2,6-dichloroanilino)phenyl]acetic acid
Structure	
Mol.mass	296.149 g/mol
Melting point	156158 °C
Solubility	In water, 2.37 mg/L at 25 °C
Partitioncoefficient (logP)	4.51
Dissociation Constants (pKa)	4.15

1.6.1 Clinical pharmacology of diclofenac (Romsing *et al.*, 2001, Stangier, 2008)

1.6.1.1 Pharmacodynamics

Diclofenac is a nonsteroidal anti-inflammatory drug which exhibits anti-inflammatory, antipyretic, and analgesic activities. Mechanism of action of diclofenac is related to prostaglandin syntheses inhibition.

1.6.1.2 Pharmacokinetics

Absorption

Diclofenac can be completly absorbed after an oral administration compared to IV administration which is measured by urine recovery. However, only about 50% of the absorbed amount is systemically bioavailable due to first-pass metabolism (Table 3). Food has no significant effect on extent of diclofenac absorption. However, there is usually a delay in absorption onset of 1 to 4.5 hours and a reduction in peak plasma levels of < 20% if the drug is administered with food.

Table 3 Pharmacokinetic Parameters for Diclofenac. (From Romsing et al., 2001, Stangier, 2008)

	Mean	**Coefficient of Mean Variation (%)**
Absolute Bioavailability (%) [n = 7]	55	40
Tmax (h) [n = 56]	2.3	69
Oral Clearance (CL/F; mL/min) [n = 56]	582	23
Renal Clearance (% unchanged drug in urine) [n = 7]	< 1	—
Apparent Volume of Distribution (V/F; L/kg) [n = 56]	1.4	58
Terminal Halflife (h) [n = 56]	2.3	48

Apparent (V/F) or volume of distribution of diclofenac is 1.4 L/kg. More than 99% of the diclofenac in blood steam can bound to those human serum proteins, primarily to albumin. Serum protein binding is constant over range of concentration (0.15 µg/mL) which can be achieved with recommended oral doses. Diclofenac diffuses into and out of synovial fluid. Diffusion into joint occurs when higher plasma levels than those in synovial fluid after process reverses and synovial fluid levels are higher than those plasma levels.

Metabolism

In human plasma and urine, five diclofenac metabolites are identified. Metabolites include 4-hydroxy, 3-hydroxy, 5-hydroxy, 4,5-dihydroxy and 3-hydroxy-4-methoxy diclofenac. For major diclofenac metabolite, 4-hydroxy diclofenac, has a very weak pharmacologic activity. 4-Hydroxy diclofenac formation is primarily formed by CPY2C9. Both diclofenac and oxidative metabolites are undergo glucuronidation or sulfation following by biliary excretion. Acylglucuronidation which can be formed by UGT2B7 and oxidative metabolism can be initiated by CPY2C8 could play a role in diclofenac metabolism. CYP3A4 is responsible for minor metabolite formation, 5-

hydroxy and 3-hydroxy diclofenac. Those patients with renal dysfunction, peak concentrations of the metabolites including 4-hydroxy and 5-hydroxy diclofenac were about 50% and 4% of the parent compound after a single oral dosing compared to 27%, and 1% in normal healthy subjects.

Excretion

Diclofenac is eliminated through metabolism and subsequent urinary and biliary excretion as glucuronide, and sulfate conjugated of metabolites. No free or little unchanged form of diclofenac is excreted in urine. Around 65% of administration dose is excreted in urine and about 35% in bile as conjugates of unchanged diclofenac plus metabolites. Since renal elimination is not a significant pathway of elimination for the unchanged diclofenac, dosing adjustment in patients with mild to moderate renal dysfunction is then not necessary. Terminal half-life of unchanged diclofenac is approximately 2 h.

1.6.2 Side effects (Baraf *et al.*, 2007)

In patients who are taking diclofenac, or other NSAIDs, most frequently reported adverse experiences occurred in approximately 1%-10% of patients have gastrointestinal effects including abdominal pain, diarrhea, constipation, dyspepsia, flatulence, gross bleeding/perforation, nausea, heartburn, GI ulcers (gastric/duodenal), and vomiting. Abnormal renal function, dizziness, anemia, edema, elevated liver enzymes, headaches, increased bleeding time, rashes, pruritus and tinnitus could also be observed as side effects in many patients.

1.6.3 Over dosage (Hunter *et al.*, 2011)

There is no significant serious clinical report, resulting from an over dosage of diclofenac. Over dosage may cause symptoms such as vomiting, gastrointestinal hemorrhage, diarrhea, dizziness, convulsions or tinnitus. However, significant poisoning, liver damage, and acute renal failure are possible in case of taking very high dose of diclofenac.

1.7 Natural Rubber (NR)

Freshly tapped *Hevea* latex contains not only rubber particles but non-rubber particles dispersed in an aqueous serum. Ratio of rubber to non-rubber components varies from source to source. *Hevea* rubber tree's rubber component is an entirely more than 98% of *cis*-1,4-polyisoprene (Figure 12) in which it is unable to crystallize under normal conditions. Therefore, it exists as an amorphous and rubbery material. Natural rubber latex (NRL) typically contains 34% (by weight) of rubber, 2%-3% proteins, 1.5%-3.5% resins, 0.51% ashes, 1.0-2.0% sugar 0.1-0.5% sterol glycosides and 55.60 % of water (Kauffman and Seymour, 1990). *Hevea* latex has pH of 6.5-7.0 and a density of 0.98 g/cm^3. Rubber particles were found varied in size from 0.15 μm to 3 μm (Dunn, 1991) and molecular weight distribution 105-107 g/mol, depending on weather, clone, tapping frequency and many factors (Westall, 1968).

The average rubber content of freshly tapped latex may be in a range of 30 to 45 percent. Fresh latex is not utilized in its original due to high water content it may contain microorganisms such as bacteria. Therefore, it is necessary to preserve and concentrate latex. Normally, the so natural rubber latex is stable and contains at least 60% of rubber. NRL concentrate is differentiated by concentration method and type of preservative used. High concentration can be achieved by centrifugation (most common) and by creaming, or by evaporation (Tantatherdtam, 2003). Ammonia is normally applied to preserve latex from bacterial attack. High ammonia (HA) latex

contains 0.7% ammonia, is still the most frequently applied to the material. 60 Percent or more of centrifuged latexes are high ammonia type.

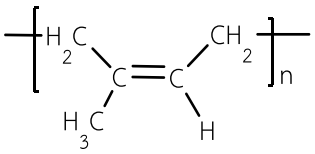


Figure 12. Structure of natural rubber (Hamzah *et al.*, 2012).

1.7.1 Natural rubber latex protein allergy

Hevea latex is considered as living cytoplasm of laticiferous cells. It contains a large number of organic substances, including a mélange of proteins. A small number of such proteins have given rise to problem of latex allergy. Proteins are about 11.5% in fresh weight of *Hevea* latex. Natural rubber latex is a non-homogeneous fluid. Therefore, latex proteins are not homogeneously dispersed. Latex proteins can be detected in latex sera. They are normally associated with latex organelles that can be separated by high-speed centrifugation. Approximately 70% of latex proteins are soluble, while remaining is being associated with membranes. Purifying of latex allergens have been described below (Yeang *et al.*, 2002).

1.7.1.1 Proteins of rubber particle

Hevea latex allergens are Hevb3 and Hevb1. These are two major proteins which located on surface of rubber particles. Hevb1 is found mainly on large rubber particles (generally above 0.4 μm in diameter) whereas Hevb3 is more abundant in smaller rubber particles.

1.7.1.2 Cserum proteins

IUIS or Union of Immunological Societies had recognized latex allergens, including Hevb5, 8, 7, and 9 are from latex C serum, which is cytosol of latex vessel. This number does not include rubber particle proteins.

1.7.1.3 Bserum proteins

To classify extracytosolic proteins in latex B serum. Lutoidic proteins B serum are prepared from “bottom fraction” which mainly consists of other organelles sediment by centrifugation is also present in the bottom fraction. Possibility that some proteins could be originate from a minor organelle which cannot be disregarded. All recognized allergenic proteins are located in the latex bottom fraction such as Hev b 2, 4, 6, and 10 which are plant defense proteins.

1.7.2 Reducing extractable and allergenic proteins in rubber latex methods (RodríguezVico *et al.*, 1989).

1.7.2.1 Leaching

Leaching or hot and cold water washing method is now utilized extensively in many dipping industries. Products thickness influence and processing conditions have been studied by many researchers. Findings are indicated as following

There is a need for protein to migrate to the rubber film surface to facilitate protein extraction.

Thinner products, are more effective of protein removal.

Latex products with a lower rubber content (40%) consists of a higher level of extractable proteins than products from latex with a higher rubber content around 60%. Wet gel leaching or leaching before curing rubber was found to have less effective than dry film leaching. This could be explained that during manufacturing process, protein had insufficient time to migrate to film surface.

1.7.2.2 Treatment of Enzyme

Use of proteolytic enzymes for digesting latex proteins is as well-known process for many years. Moreover, usage of these enzymes has been shown recently to reduce allergenic proteins in medical gloves. These enzymes can destroy protein by break up proteins into smaller pieces. They facilitate their removal. In order to remove residual proteolytic enzymes, which may give a rise to an allergic response, have to be taken with care.

1.7.2.3 Fumed silica

It is theorized that fumed silica can be used in both straight and coagulant dipping procedures in order to reduce extractable protein in surgical gloves. It is claimed that as little as 12 part per hundred of dry rubber (phr) can significantly reduce extractable protein from those latex gloves without affecting adversely dipping process.

1.7.2.4 The leaching of ultrasonic

There are reported that using ultrasonic in leaching system can accelerate latex protein removal over leaching on its own. Effect is likely to be more pronounced in pre-vulcanized films than in post-vulcanized films. There is apparently no significant effect on tensile properties of the rubber films.

1.7.2.5 Chlorination

Primary purpose of chlorinating rubber latex in medical gloves preparation is to provide non lubricating powder gloves. Chlorination is found to be one of the method that show much more effective in lowering level of extractable protein. The process requires tight controls since over use of chlorination or inadequate neutralization of powerful chemicals can lead to detrimental and discoloration effects on ageing properties of gloves.

1.7.2.6 Miscellaneous

There are many alternative approaches that have received much attention for example. Incorporation of water soluble polymers into radiation

vulcanized latex films. High pressure treatment of natural rubber latex as well as ozone water treatment of latex are usually known procedure in protein removing methods.

1.8 Epoxidized natural rubber

As described earlier that many methods in chitosan films and microspheres formulation were reported. Films and microspheres of chitosan could be prepared by using a crosslinking agent such as ethylene glycol diglycidyl ether (Figure 13) or in combination with sodium tripolyphosphate (Mi *et al.*, 2002). However, these crosslinking agents are expensive and available only from foreign countries. Common natural rubbers originated from plant *Hevea brasilensis.* Major uses of natural rubber are for tire making, molded goods and mechanical parts. Lesser uses of natural rubber are rubber chemical derivatives. However, property improvement of natural rubber for certain applications can be done via chemical modifications. This is possible as presence of double bonds (C=C) in natural rubber polymer chain (Figure 12) act as simple olefin. One of important chemical modification products of natural rubber is epoxidized natural rubber, ENR. Natural rubber with peroxy formic acid can provide ENR (Fig. 13a and b). Normally, 190% epoxidation of natural rubber could be possible but however only 3 types of ENR are considered as commercial standard. These are ENR-10, ENR-25, and ENR-50 while integers designate to 10, 25 and 50 mole % of epoxide incorporated to natural rubber chain respectively. According to chemical modification of natural rubber, some properties will be enhanced. ENR displays properties that are similar to elastomer such as decrement in air permeability. It is comparable to a butyl rubber which shows increment in oil resistance, which is comparable to acrylonitrile butadiene rubber (ABR) (Yoksan, 2008, Hamzah *et al.*, 2012).

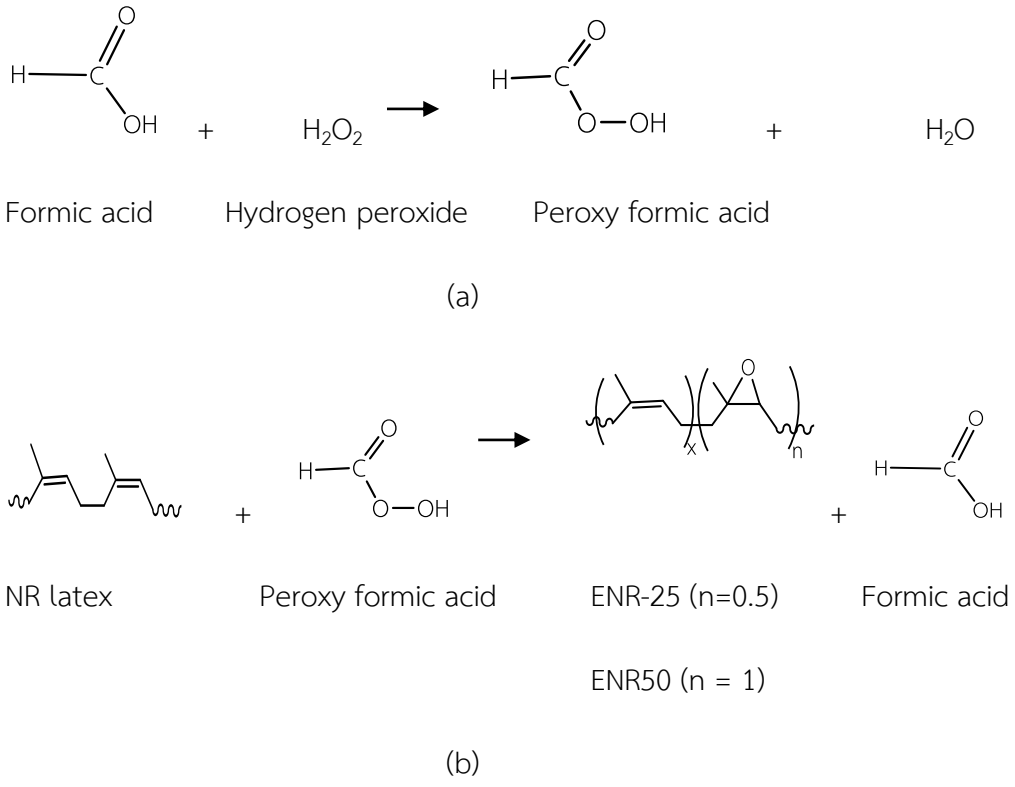


Figure 13. (a) Formation of peroxy formic acid and (b) reaction to produce ENR (Yoksan, 2008).

ENR can be used in modification of polymer and blending by reacting together with common epoxy curing agents and chemicals which contain OH, NH, COOH, and C=C groups, etc. Few applications of ENR can be derived from this great reaction versatility such as epoxidized natural rubber (ENR) latex which has 25% of epoxide content. It was prepared by *in situ* epoxidation reaction, using performic acid. ENR latex film surface had modified by immersing into methyl methacrylate (MMA) emulsion, follow by alkaline aqueous solution of ferrous ion/fructose for redox initiated polymerization. Increasing polymerization time caused percent conversion of MMA swelled in ENR sheet increase (Amornchaiyapitak *et al.*, 2008). Bovine gelatin based films modified with epoxidized natural rubber with different epoxy contents (ENR-10, ENR-25 and ENR-50) were prepared by solution casting. Result indicated that increasing epoxy content and concentration of ENR used, tensile strength (TS), UV and visible light transmission of films decreased but elongation at break (EAB) representing

film stretch ability/flexibility and yellowness of films increased (Chamnanvatchakit *et al.*, 2009). Effects of epoxidized natural rubber (ENR-50) as a compatibilizer on styrene butadiene rubber or recycled acrylonitrilebutadiene rubber (SBR/NBRr) blend properties were studied. Additional study of ENR-50 in SBR/NBRr blends, and better adhesion between SBR and NBRr was found. compatibility of SBR/NBRr blends was improved (Noriman *et al.*, 2010). Moreover, ENR-25 and ENR-45 were blended with cassava starch in latex state. Pure ENR exhibited lower shear viscosity and shear stress than those blends with cassava starch. In addition, shear stress and shear viscosity were increased with an increase in concentration of cassava starch. Chemical interaction between epoxide groups in ENR and hydroxyl groups in cassava starch molecules could be reason for increasing trends of shear viscosity and shear stress (Nakason *et al.*, 2004).

Therefore, the objective of this study is to investigate preparation methodology for preparation chitosan microspheres and films by cross-linking reaction using epoxidized natural rubber and utilized the resulting films and microspheres for drug delivery.

CHAPTER 2

CHITOSAN FILMS

2.1 INTRODUCTION

These days' biodegradable polymers from renewable sources are gaining more interest due to environmental serious issues. Film are the materials among other materials that are in high demand due to their variety of applications. Films can be prepared by using polymers which are dependent on polysaccharides, proteins and lipids. They are usually biodegradable and harmless in nature. They can be used in many applications. Therefore, a growing interest to develop films were noticed. Several polymers materials were used to investigate for their ability in films formulation process. Chitosan is an interesting film forming material that has been focused in many research groups (Leceta *et al.*, 2013).

Chitosan can be employed as biomaterial in films preparation with the help of casting technique which makes it a valuable tool for many biomedical uses such as skin care products (Boucard *et al.*, 2007), cosmetics (Silva *et al.*, 2013), contact lenses, membranes (Shenvi *et al.*, 2013) and separators (Narayanaswamy and Prabhu, 2013). Chitosan films are usually clear, smooth, and flexible in nature and have potential as oxygen barrier. (Hosokawa *et al.*, 1990). Chitosan films are dense and free from pores. It is known that the characteristics of chitosan films is dependent on their morphology and depends upon molecular weight of chitosan source, degree of deacetylation, technique of film development, and on the nature of solvent that will be employed for dissolving chitosan. (El-hefian *et al.*, 2009). The usage of acetic acid is usually recommended for the

solubility of chitosan and film formation technique. For example, Shu *et al* (2001) reported the preparation procedure by using 4% w/v chitosan in 4% v/v aqueous acetic acid followed by a casting/solvent evaporation technique. Casting chitosan films by using other acid solutions such as citric, lactic, and malic acid without plasticizer was performed. Tensile strength and elongation of the obtained films ranged from 6.7 to 150.2 MPa and from 4.1 to 117.8%, respectively. The result demonstrated that the tensile strength increased with increasing in chitosan molecular weight. Toughest films can be developed by the usage of acetic acid as compared to the usage of malic, lactic, and citric acid, respectively. Films prepared with citric acid had the highest elongation values (Park *et al.*, 2002). Thermoplastic starch film was prepared by incorporating chitosan as a plasticizer with a content of 0.73, 1.09 and 1.45%, (w/v) respectively. The resulting films possessed better extrusion ability, increased tensile strength, rigidity, thermal stability and UV absorption, as well as reduced water absorption and surface stickiness (Dang and Yoksan, 2015).

2.1.1 Techniques for films of chitosan preparation

Chitosan is usually purchased from the market in the form of dried flakes. Dilute hydrochloric acid and acetic acid are considered the best solvents for preparation of the chitosan solution of these flakes and then filtered to remove particulates. Several different methods for construction of chitosan film have been developed and reported and solution casting method is one of the most convenient method that is widely used. This process, chitosan is dissolved in suitable solvents, in most cases slightly acidified water, and a plasticizer is added. The solution is poured on a flat surface and the solvent is allowed to evaporate. A comprehensive research was performed by van den Broek *et al*, 2014 in which the solvent and pH of the solution was varied for low (78.9%) and high (92.3%) degree of de-acetylation (DDA) of the chitosan material were employed. The main

focus of the water vapour permeability (WVP) of the obtained films was investigated. The WVP was significantly affected by the DDA of chitosan. Higher WVP was measured for highly de-acetylated chitosan than for chitosan with lower DDA.

During the casting process, the solvent was evaporated from the chitosan solution and a thickness of the films formulations is depended on the concentration of the solution matrix. Elevated temperatures can be used to speed up the solvent evaporation. The resulting chitosan films can be utilized in many applications especially in pharmaceutical products. Nowadays, it is commonly appied for the production of engineering plastics, optical films, medical films and sheet forming for electronic applications.

Several factors have been investigated for their effects in chitosan film forming process (Siemann, 2005). Since, non-soluble materials may provide the un-desired surface morphology of the film. Moreover, a stable and optimum viscosity of the chitosan solution is required to provide good appearance of the film and easy to remove the film from the casting support. Therefore, in a number of research investigations in optimization for suitable type of co-polymers and additives including plasticizers and releasing agent were performed.

For example, Boston and co-workers (2014) prepared chitosan films blended with polyethylene oxide by solution casting method. Their physico-chemical characterizations were determined by using FT-IR, DSC, XRD and SEM. The results suggest that the blended films have homogenous surface. Chitosan in the films were in amorphous state with intermolecular hydrogen bonding between the blend components. Another work was reported on the preparation of chitosan-polyvinyl alcohol blend film by casting method (Kanatt *et al.*, 2012). Mint extract was added in the mixture before casting. The resulting film composites were homogenous and can be easily peeled from the casting devices. The films displayed high tensile strength (41.07≈ MPa) which may resulting from the strong inter-molecular hydrogen bonding between chitosan and polyvinyl alcohol molecules. Chitosan films containing naringin, the bioactive citrus extract were obtained by solvent

casting evaporation method. DSC results suggested the cross-linked between naringin and chitosan occurred and that made the decrease in releasing of naringin from the irradiated samples (Iturriaga *et al.*, 2014).

Many researches were performed to prepare chitosan containing an active aroma compounds, carvacrol for delivery of active volatiles. Chitosan blended with hydroxyl propyl-β-cyclodextrins films were prepared by casting method. The resulting films were subjected to immerse in carvacrol solution for 3 weeks to reach sorption equilibrium. The films containing carvacrol were transparent, yellow color and no discontinuities (Higueras *et al.*, 2014). Glycerol were additionally added to the former formulation for chitosan films formulations and the resulting films were soaked in liquid carvacrol. The results demonstrated that the films showed antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* even after 20 days of storage. Therefore, the films could absorb and also release the carvacrol (Higueras *et al.*, 2015). The physico-mechanical properties of the chitosan films mixed with carvacrol and grape seed extract were determined and reported (Rubilar *et al.*, 2013). The solvent casting method was selected for preparation of the films. The carvacrol, grape seed extract loaded into the chitosan films including film-1: 9.6 ppm–684 ppm–1.25% w/v and film-2: 90 ppm–160 ppm–1.24% w/v, respectively and compared with film control (1.25% w/v chitosan). Mechanical, structural and barrier properties of the films were characterized. Film-2 presented the lowest water vapor and carbon dioxide permeability and tensile strength values and the highest oxygen permeability, whereas film-1 showed the highest water content and the lowest crystallinity, carbon dioxide permeability and tensile strength. These results recommend that carvacrol and grape seed extract in the selected range affect the film structure and its mechanical properties because hydrophilic (grape seed extract) and hydrophobic (carvacrol) compounds.

Chitosan films were of interest to develop for buccal drug delivery to many researchers. Mucoadhesive chitosan/gelatin blend films containing propranolol hydrochloride were prepared by casting method (Abruzzo *et al.*, 2012). The FT-IR and TGA

analysis results confirmed the interaction between chitosan and gelatin. Increasing amount of chitosan in the film formulations resulted in lowering water uptake ability but improving the *in vivo* buccal cavity residence time. Chitosan films containing Ibuprofen for oral mucosal drug delivery were developed (Tang *et al.*, 2014). The SEM and X-Ray diffraction results revealed that Ibuprofen in the films were in microparticles, flakes, rod-like and needle-like crystals. The *ex-vivo* release study using rabbit buccal mucosa found that up to 70% of Ibuprofen could release from the film matrix in slow release characteristic within 460 mines.

Several other methods for chitosan films preparation have been reported and the preparation procedures could be useful for biomedical devices preparation such as electrospinning, printing and electrodeposition.

Preparation of chitosan films by electronspinning.

Nanofibers are usually generated by electrospinning technique. This technique has demonstrated to be the low cost method and usually give nanofibers which have suitable properties. Nanofibers have gained a lot of attentions from the last couple of decades because their usage as a nanoscale is very attractive (Ziani *et al.*, 2011). Electrospun nanofiber mats are normally flexible, highly porous and permeable materials that provide a high surface-to-volume ratio ideal for wound dressings. The well-established technique of electrospinning has been utilized to produce non-woven mats from over 100 different synthetic and natural polymer solutions (Rieger and Schiffman, 2014). Novel hybrid nanofibers were constructed by using chitosan containing a total amount of polyethylene oxide and silica precursors by electrospinning. The tunable properties of these hybrid nanofibers are suitable to use as active biomaterials in bone repair and regeneration (Toskas *et al.*, 2013). Chitosan nanofibers cross-linked with genipin were prepared by electrospun. The average fiber diameters of the electrospun were 227$\pm$154 nm as spun, and increased to 335$\pm$119 nm after cross-linking with genipin. The Young's modulus of

the composite nanofibrous was 142 $\pm$ 13 MPa (Frohbergh *et al.*, 2012). Chitosan/sericin/poly(vinyl alcohol) as a biodegradable nanofibrous membrane was prepared through electrospinning. The result showed that the nanofibers were small diameter and narrow size distribution without beads. The mean diameter of nanofibers was about 180 nm (Hadipour-Goudarzi *et al.*, 2014). Moreover, antimicrobial of nanofibrous membranes were observed by electrospinning of chitosan/poly(ethyleneoxide) incorporation with poly(hexamethylene biquainde) hydrocloride. The morphology of electrospun nanofibers demonstrated the average size of electrospun structure of fiber was 153 nm (Dilamian *et al.*, 2013).

Printing

Nanoimprinting and microcontact printing have been used successfully for developing chitosan films with nanoscale resolution. In these techniques a patterned mold or stamp is employed which is usually a flexible polymer such as chitosan. Printing techniques can develop a vast number of chitosan structures for spatially resolved functionalization. Nanoimprint lithography and microcontact printing both are unique planar processes and their usage for chitosan films on non-planar surfaces are challangable. Alignment of the mold or stamp to develop preexisting characteristics on the substrate is usually very difficult (Koev *et al.*, 2010).

Electrodeposition

Electrodeposition is a versatile and efficient approach for fabricating films (Boccaccini *et al.*, 2010). It is a process in which an imposed electric field is applied on charged particles which are dispersed in a liquid towards an electrode for the synthesis of thin films. Electrodeposition has advantages of short processing time, room temperature processing, and no requirement of cross-linking agents. The coatings or films prepared with the method of electrodeposition have been widely used in the biomedical and

biotechnology fields (Wang *et al.*, 2014). Lei *et al* (2011) have developed biosensing from 3-aminopropyltriethoxysilane-chitosan hybrid gel by electrodeposition. The 3-aminopropyltriethoxysilane-chitosan film showed good porosity and good film-forming ability of chitosan hydrogel film, which should be suitable for robustly entrapping biomacromolecules in the biocompatible hybrid gel film. High loading as well as highly active material making the electrode substrate still sufficiently electroactive for biosensing due to the presence of many unoccupied electrode sites after deposition of the porous gel film. Cheng et al (2012) reported cathodic electrodeposition method of semicrystalline chitosan hydrogel. Synchrotron X-ray studies on the hydrated electrodeposited gels indicated the semicrystalline structure of neutralized chitosan was observed. The results was described that the crystalline structure of chitosan and proposed an electrodeposition mechanism as resulting from the formation of interconnected semicrystalline microgels to form the electrode.

From the previous mention, chitosan films can be easily prepared by chemical cross-linking reaction and EGDE is one of the common cross-linking agent that are being used. EGDE has two epoxide rings at its terminal (Lu *et al.*, 2006) and has been utilized as a common cross-linking agent for a number of biological polymers for example gelatin, (Almeida *et al.*, 2007, Vargas *et al.*, 2008) collagen, (La Gatta *et al.*, 2013) keratin, (Tanabe *et al.*, 2004) as well as chitosan (Ngah *et al.*, 2005, Kouketsu *et al.*, 2007, Ozcelik *et al.*, 2013). It is known as a cross-linker for carboxyl-, amine- and hydroxyl- functional groups in the preparation of polymers. In a cross-linking reaction between chitosan and EGDE, the amine groups of chitosan were nucleophiles, that reacted by attacking at the more substituted carbon of the epoxide ring, to make a ring opening and resulted in a chitosan cross-linked polymer (Kurmaev *et al.*, 2002). Chitosan films prepared by the cross-linking reaction between chitosan with EGDE provided films with good conformity and microstructure with a high tensile modulus and decreased the ultimate elongation without affecting the maximum stress (Ghosh *et al.*, 2010). Macro porous chitosan membranes with controlled pore size and good mechanical properties were previously prepared by the

cross-linking reaction between chitosan and EGDE and were used for protein separations in the ion-exchange mode (Zeng and Ruckenstein, 1998). Even though, EGDE is one of the most commonly used cross-linking reagent, it is still expensive and therefore, results in a high cost of the film. Natural rubber is a product from the latex of the rubber tree, *Hevea brasilensis* (Kohjiya and Ikeda, 2014). The major uses of natural rubber are for tires production, molded goods, and mechanical parts. Properties of natural rubber for specific applications can be produced via chemical modifications (Phinyocheep, 2014). This is possible because of the presence of large volume of double bonds (C=C) in the natural rubber polymer chain which act as a simple olefin. One of the important chemical modifications of natural rubber is to form epoxidized natural rubber or ENR. It is derived from a partial oxidation of the natural rubber molecule. The epoxide groups are randomly distributed (10-50%) along the chain of the natural rubber molecule (Yu *et al.*, 2008). ENR can be utilized in polymer modification by reacting with common epoxy curing agents and any compounds containing functional groups such as -OH, -NH_2, -COOH, and -C=C groups, and etc. A number of new applications of ENR could be derived from this great reaction versatility (Gan and Abdul Hamid, 1997, Hong and Chan, 2004, Yoksan, 2008). It has been utilized for reactive blending with starch and other polymers having polar functional groups to improve their physico-chemical properties (Nakason *et al.*, 2001, Nakason *et al.*, 2003, Wu *et al.*, 2004, Johns and Rao, 2009, Tanrattanakul and Chumeka, 2010, Ismail *et al.*, 2011). A number of works on the preparation of chitosan-filled rubber composites have been previously reported in which the main component was ENR or equal amounts of ENR and chitosan (Letwattanaseri *et al.*, 2009, Shaari *et al.*, 2011, Sukhlaaied and Riyajan, Riyajan and Sukhlaaied, 2013).

However, ENR has never been used as a cross-linking agent in the formation of chitosan films. Since the cross-linking reaction of chitosan with an EGDE cross-linker occurs via nucleophillic ring opening, reaction of the amino groups of chitosan with the epoxide ring of EGDE, epoxidized natural rubber could be used to replace EGDE in chitosan film

preparations. The chemical cross-linking reaction could be performed in a similar manner to that described for EDGE.

For example, chitosan biomaterials were prepared by ENR grafted chitosan reported by Riyajan and co-worker in (2013) using potassium persulfate as radical initiator in the grafting reaction. The reaction start from sulfate free radical generated from potassium persulfate then sulfate free radical will degrade chitosan chain to produce two shorter chains. One has a free radical on C-1 carbon and another has carbonyl at C-4 carbon. The chitosan free radical chain will react with ENR molecule by ring opening reaction of epoxide to from ENR fee radical. At same time another short chitosan chain with carbonyl group will react with ENR free radical to form co-polymer (Figure 14). In this work, chitosan grafted ENR was obtained by using free radical reaction which may have some initiator remaining and may not good for skin application.

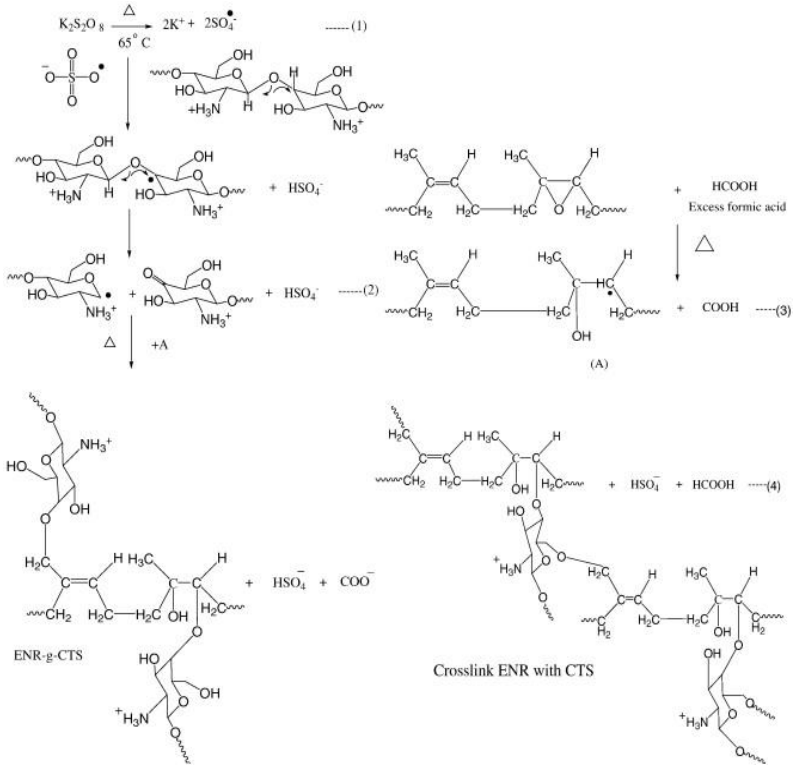


Figure 14. Proposed reaction mechanism in ENR-grafted-chitosan films reported by Riyajan *et.al* (2013).

Another work of chitosan grafted ENR preparation was reported by Haris and co-worker (2014). This work chitosan grafted on to backbone ENR was performed by using $AlCl_3.6H_2O$. The proposed the reaction mechanism for the formation of chitosan grafted ENR are displayed in Figure 15. First after mixing chitosan and ENR-50 with $AlCl_3.6H_2O$, partially protonated chitosan and protonated ENR were obtained. Then the primary amino groups of the chitosan will attack on the protonated epoxidized isoprene unit at more stearic carbon of epoxide ring, resulting in chitosan grafted-ENR as a major product. The product of this reaction may not also suitable for biomaterial application since aluminum may remain in the final product. From those two reports in preparation of chitosan grafted ENR, ENR was used as a major component whereas, chitosan was used as cross-linker. In this work we would like to prepare chitosan films by using chitosan as a major component for biomaterial application and use ENR as a cross-linker. Therefore purifying ENR by removing any protein that may remain in the ENR before using was performed. This is the different from the previous works that they used ENR without any deproteination. Chitosan was prepared by using acetic acid solution as a medium which is a nontoxic compound. Therefore, the objectives of this study are:

1) To find the optimum conditions for the preparation of chitosan films by cross-linking reaction using deproteinated epoxidized natural rubber.

2) To characterization of physicochemical properties of the chitosan films (with drug and without drug).

3) To evaluate the drug loading and the *in vitro* release of the model drug from the chitosan films.

4) To study skin irritation of the obtained chitosan films.

5) To study stability of the model drug from the chitosan films.

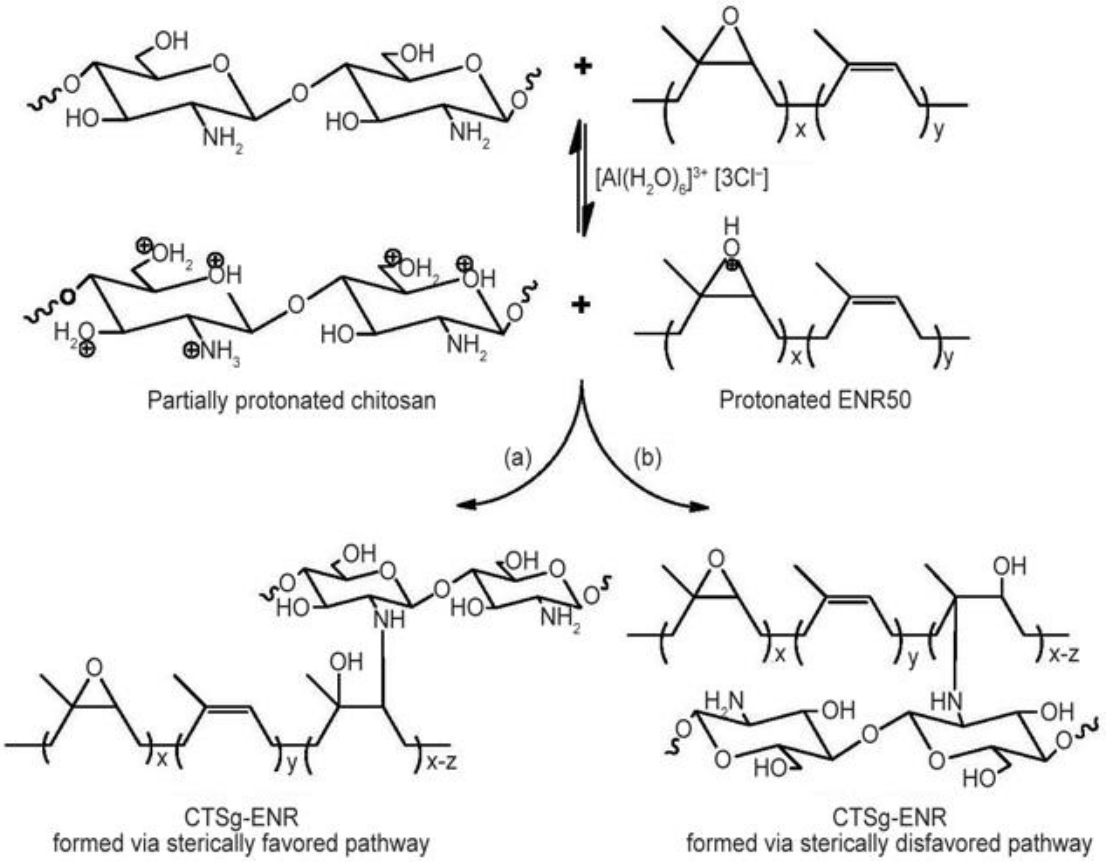


Figure 15. Proposed reaction mechanism in ENR-grafted-chitosan films reported by Haris *et.al* (2014).

2.2 EXPERIMENTAL

2.2.1 Chemicals

The reagents and substance used in the study are listed as the following:

- Acetic acid, analytical grade (Labscan Ltd., Bangkok, Thailand)
- Acetic acid-D4, NMR grade (Cambridge isotope laboratories, Inc., USA)
- Albumin bovine serum, analytical grade (Acros organics, USA)
- Chitosan (mw = 300,000 and %degree of deacetylation = 80) (Seafresh Chitosan (Lab) Company Limited, Thailand)
- Chloroform-D, NMR grade (Cambridge isotope laboratories, Inc., USA)
- Copper (II) sulphate, analytical grade (Ajax Finechem Pty Ltd., Australia)
- Deuterium oxide, NMR grade (Cambridge isotope laboratories, Inc., USA)
- Diclofenac (raw material, China)
- Dichloromethane, analytical grade (Labscan Asia co., Thailand)
- Dimethyl sulfoxide, analytical grade (Labscan Ltd., Bangkok, Thailand)
- Epoxidized natural rubber, 25 and 50 mole% epoxidation levels (ENR-25 and ENR-50) (Muang Mai Guthrie Co. Ltd., Thailand)
- Ethylene glycol diglycidyl ether (TCI America, USA)
- Folin-ciocalteu's phenol reagent (Fluka®, Switzerland)
- Methanol, analytical grade (Labscan Ltd., Bangkok, Thailand)
- Polyethylene glycol 200 (Merck®, Germany)

- Potassium dihydrogen phosphate, analytical grade (Ajax Finechem Pty Ltd., Australia)
- Potassium tartrate, analytical grade (Ajax Finechem Pty Ltd., Australia)
- Sodium carbonate, analytical grade (Rankem, India)
- Sodium chloride, analytical grade (Labscan Ltd., Bangkok, Thailand)
- Sodium dihydrogen phosphate dihydrate, analytical grade (Guangdong, China)
- Sodium hydroxide, analytical grade (Labscan Ltd., Bangkok, Thailand)
- Tetrahydrofuran, analytical grade (Labscan Ltd., Bangkok, Thailand)
- Toluene, analytical grade (Labscan Ltd., Bangkok, Thailand)
- Trichloro acetic acid, analytical grade (Sigma-Aldrich, Germany)
- Tungstophosphoric acid hydrate, analytical grade (Merck®, Germany)
- Water was purified in a Milli-Q system (Millipore, Bedford, MA, USA)

2.2.2 Instruments and equipment

- Analytical balance (Sartorius® model, BSA 2248, Scientific Promotion co., Ltd., Thailand)
- Chromatographic Column (C8 reverse-phase column, 150 mm x 4.6 mm; particle size 5 µm; ACE®, Scotland)
- Differential scanning calorimeter (Perkin Elmer, DSC7, USA)
- Fourier transforms infrared spectrometer (Perkin Elmer, Spectrum one FT-IR spectrometer, USA)

- High performance liquid chromatography (Agilent 1100 series, Germany)
- Hot plate and magnetic stirrer (Cemarec®2 Thermolyne SP46920-33, USA)
- Micropipette 1000 and 200 µL (Biorad®, USA)
- Modified Franze diffusion cell (model 57-951-061, Hanson Research Corporation, USA)
- Nuclear Magnetic Resonance (Fourier transform NMR spectrometer 500 MHz, Unity Inova, Varian, Germany)
- pH meter (Seven Easy S-20, USA)
- Ultrasonic bath (model DSC 104, D.S.C. group co., Ltd., Thailand)
- Universal testing machine (For tensile strength, elongation at break determination), model NRI-TS500-25, Thailand)
- UV-visible spectrophotometer (Hewlett Packard 8452A, Diode Array Spectrophotometer, USA)
- X-ray diffractometer (X'Pert MPD, Philips analytical, Netherlands)

2.3 METHODS

2.3.1 Purification and characterization of epoxidized natural rubbers

2.3.1.1 Purification of epoxidized natural rubbers

Epoxidized natural rubbers were purified by method of (Klinpituksa, 1991, Siler and Cornish, 1995, Yeang *et al.*, 1995) with slightly modified. Epoxidized natural rubbers 5g was dissolved in toluene (100 mL) at room temperature (30±1 °C) with magnetic stirrer at 500 rpm for 24 h. Purified epoxidized natural rubber was obtained by precipitation using methanol. The precipitate was washed with distilled water. The result was added in the warm water (60-80 °C) for 4 h and then dried in an oven at 40 °C until a constant weight was obtained. The process was repeated if any protein was still found in the ENR product.

2.3.1.2 Proteins content determination of purified epoxidized natural rubbers

Content of protein in purified epoxidized natural rubbers was determined by method of Beezhold et al (2002) with slightly modification. Protein extracts were prepared from epoxidized natural rubber as following. ENR-25 or ENR-50 (1g) was cut into small pieces, soaked with 30 mL of deionized water at 37 °C for 3 h. The sample was centrifuged at 3000 rpm for 20 min to remove insoluble matter before measurement of protein concentration in supernatant by the Lowry method. (Lowry *et al.*, 1951), as following procedure.

The supernatant (30 mL) was mixed with 3 mL of freshly prepared alkaline copper solution (the mixture of 2% Na_2CO_3 in 0.1 N NaOH, 1% potassium tartrate and 0.5% $CuSO_4.5H_2O$ at the ratio of 100:1:1). After mixing, the mixture was left standing at room temperature for 10 min. 0.1% Folin-Ciocalteu's phenol reagent (0.3 mL) was then added, mixed thoroughly and left standing at room temperature (30±1 °C) for 30 min. After that

the samples were measured the absorbance at 650 nm. Absorbance readings, recorded at 650 nm, were calibrated against standard bovine serum albumin (BSA). Protein content in samples were calculated by comparison with standard curve of bovine serum albumin (BSA) prepared in the same manner. Results were expressed in μg/g of the ENR samples. The protein was calculated using the following equations, (ISO12243:2003, 2003).

$$C = (c \times M_w) / (M_p \times 6)$$

When

C = Concentration of protein (μg/g)

c = weigh of protein (μg), calculate form (1/slope) x Absorbance

M_p = weigh of ENR (g)

M_w = weigh of water for extraction proteins (g)

2.3.1.3 Characterizations of deproteinated epoxidized natural rubbers

The ^{1}H NMR spectra were measured on NMR 500 MHz, using 5 mg of ENR and dissolved in $CDCl_3$. %Epoxidation was calculated from the ^{1}H-NMR spectra using the equation below (Saito *et al.*, 2007). Apparent molecular weights and molecular weight distribution of polymers were determined by GPC system (Shimadzu), with a computer control dual pump, a RID-10A refractive index detector. A Shodex GPC KF-806M (8.0 mm ID x 300 mm length) and shodex GPC KF-803L (8.0 mm ID x 300 mm length) in series were columns used in the system. Tetrahydrofuran (THF) was used as an eluent, and the flow rate was 1.0 mL/min, at 40 $^{\circ}$C. Standard polystyrenes were used for calibration.

$$\% \text{ epoxidation} \frac{I_{2.70}}{I_{2.70} + I_{5.14}} \text{x } 100$$

Where $I_{2.70}$ and $I_{5.14}$ are integrated area of peaks at chemical shifts 2.70 and 5.14 ppm corresponding to epoxidized hydrogen and olefin hydrogen of natural rubber, respectively.

2.3.2 Optimization for a suitable ratio between type of solvent and deproteinated epoxidized natural rubbers for cross-linking reaction.

Since, deproteinated ENR will be added to chitosan solution in a solution form during cross-linking reaction process, therefore the suitable solvent to dissolve is required. The solvent including dichloromethane, dimethylsulphoxide, tetrahydrofuran, and toluene were evaluated as followed: deproteinated dP-ENR-25 or dP-ENR-50 (1g), was cut into small pieces (0.2 x 0.2 cm) and soaked with 100 mL of each selected solvent, and then stirred at room temperature for 24 h. The suitable solvent should give clear solution after mixing without observation of any precipitation.

Since, the result from previous investigation demonstrated that tetrahydrofuran and toluene were the best solvents to dissolve both deproteinated-ENRs. However, toluene will not be chosen to be used in the next section since toluene was not miscible with water. Hence, the next step of the investigation was to evaluate for the optimum amount of THF for dissolving deproteinated ENRs. (0.1, 0.2, 0.5, 1, 2, 3, 4, 5, and 10 g) of deproteinated dP-ENR-25 or dP-ENR-50 was cut into small pieces (0.2 x 0.2 cm) and then soaked with 100 mL of THF and stirred at room temperature (30 ± 1 ^{o}C) for 48 h. The optimum amount of solvent was selected from the ENR solutions that can give clear solution after mixing without observation of any precipitation.

2.3.4 Optimization for protocol to prepare chitosan films

2.3.4.1 Optimization for a suitable concentration of deproteinated epoxidized natural rubbers.

The suitable amount of deproteinated dP-ENR-25 or dP-ENR-50 that will be used as cross-linking agent in films formulations were determined using the following procedure. Chitosan solution was prepared by dissolving 1.50 g in 1.0% acetic acid solution (100 mL) at room temperature (30±1 °C) with magnetic stirrer for 24 h. Solution (1.0 mL) of deproteinated dP-ENR-25 or dP-ENR-50 (0.10, 0.25, 0.50, 1.00, 2.00, 3.00, 4.00 and 5.00 g/100 mL) in THF were added to the chitosan solution and stirred at room temperature (30±1 °C) for 24 h. The suitable ratio concentration of the ENR solution were selected from the solution that gave clear solution without observation of any precipitation.

2.3.4.2 Optimization for a suitable volume of deproteinated epoxidized natural rubbers (dP-ENR).

The suitable volumes of dP-ENR-25 or dP-ENR-50 that will be used as cross-linking agent in films formulations were determined using the following procedure. Chitosan solution was prepared by dissolving 1.50 g in 1.0% acetic acid solution (100 mL) at room temperature (30±1 °C) with magnetic stirrer for 24 h. Solution of 1.0 % of dP-ENR-50 or 4.0% of dP-ENR-25 (0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 2.00, 2.50 and 3.00 mL) in THF was added to the chitosan solution and stirred at room temperature for 24 h. A suitable volume of dP-ENR was selected from the maximum volume which will gave clear solution without precipitation.

2.3.4.3 Optimization for a suitable concentration of chitosan solution for film formulation.

Different concentrations of chitosan solutions were optimized for chitosan films preparation including 1.00, 1.25, 1.50, 1.75, 2.00 and 2.25 g (w/v) in 1.0% acetic acid solution. Each solution (100 mL) was prepared at room temperature (30±1 °C) with

magnetic stirrer for 24 h. Solution (1.0 mL) of 1.0% dP-ENR-50 or 4.0% dP-ENR-25 in tetrahydrofuran was added to the chitosan solution and stirred at room temperature for 24 h. The resulting mixtures were casted on a petri dish and spread manually to the outside borders. The films were dried in an oven at 40 °C for 48 h. The suitable formulation were selected from the composition which gave clear solution without any precipitation or any aggregation after mixing.

2.3.5 Preparation of blank chitosan films.

Three types of blank chitosan films were prepared and will be used for comparison study to that the films containing drug.

2.3.5.1 Blank chitosan film without cross-linker

Blank chitosan films without cross-linker were prepared by dissolving chitosan 1.50 g in 1.0% acetic acid solution (100 mL) at room temperature (30±1 °C) with magnetic stirrer for 24 h. The solution was then casted on a petri dish and spread manually to the outside borders. The films were dried in an oven at 40 °C for 48 h.

2.3.5.2 Blank chitosan films cross-linked with dP-ENR

Blank chitosan films cross-linked with dP-ENR were prepared by dissolving chitosan 1.50 g in 1.0% acetic acid solution (100 mL) at room temperature (30±1°C) with magnetic stirrer for 24 h. Solution of 4.0% dP-ENR-25 or 1.0% dP-ENR-50 in tetrahydrofuran (1 mL) was added to the chitosan solution and continued stirring at room temperature (30±1 °C) for 24 h. The resulting mixtures were then casted on a petri dish and spread manually to the outside borders. The films were dried in an oven at 40 °C for 48 h.

2.3.5.3 Blank chitosan cross-linked with Ethylene glycol diglycidyl ether (EGDE)

Blank chitosan films cross-linked with EGDE were prepared by dissolving chitosan 1.50 g in 1.0% acetic acid solution (100 mL) at room temperature (30±1 °C) with magnetic stirrer for 24 h. Solution of EGDE (0.54 mL, 0.0035 mol) was added to the chitosan solution and continued stirring at room temperature (30±1 °C) for 24 h. The resulting mixtures were casted on a petri dish and spread manually to the outside borders. The films were dried in an oven at 40 °C for 48 h.

2.3.6 Physicochemical characterization of blank chitosan films

2.3.6.1 Morphology observations

The surface morphologies of blank chitosan films cross-link with dP-ENR-25, dP-ENR-50 and EGDE were examined using scanning electron microscopy (SEM, Quanta400, FEI, Czech Republic). Prior to observation, samples were mounted on metal grids, using double-sided adhesive tape, and coated by gold under vacuum before observation.

2.3.6.2 Film swelling

The swelling behavior of the dried film samples (1 × 1 cm^2) with a thickness about 30 μm was determined in phosphate buffer saline solution at pH 7.4 at 25 °C. The dried films were weighted and conditioned in the buffer solution until the equilibrium swelling is reached (48 h). The film samples were taken from the medium, wiped with a filter paper and weighted. The swelling property was reported as %swelling by using the following equation for calculation. The test was performed in triplicate.

$$\%\text{Swelling} = \frac{(W_t - W_0)\ x\ 100}{W_0}$$

where W_t and W_0 represent the weight of swollen and dried state samples, respectively.

2.3.6.3 Moisture uptake

Moisture uptake property of the film was measured using the method similar to the previously reported method (Amnuaikit *et al.*, 2005) with some modification. The film samples (1 cm x 1 cm) were put in a desiccator containing anhydrous silica gel for 24 h and weighed (W_s), the films were then transferred to another desiccator containing saturated NaCl solution (relative humidity 75%) at 25°C. After equilibrium was reached, the films were taken out and weighed (W_m). Moisture uptake capacity was calculated according to the following equation:

$$\text{Moisture uptake capacity} = \frac{(W_m - W_s)x\ 100}{W_s}$$

where W_s is weight of the film at initial and W_m is weight of the film after equilibrium of moisture.

2.3.6.4 Film thickness

The film thickness was determined using a digital micrometer (SM-114, Teclock, Japan). Ten thickness measurements were randomly taken throughout the film on each testing sample. The mean values were calculated and used for tensile strength (TS) measurement.

2.3.6.5 Mechanical properties

Tensile strength (TS) and elongation at break (EAB) of the films were carried out according to ASTM D412 C standard on a universal testing machine (model NRI-TS500-25, Thailand). The specimen was tested at a crosshead speed of 10 mm/min at 25°C. Dog bone shaped of film samples (30 × 4.5 × 0.3 mm^3) were conditioned in a chamber having 65 ± 2% HR before testing. The tensile strength and elongation at break were calculated using the following equation using the average values of triple replicates.

$$\text{Tensile strength (TS)} = \frac{\text{load at break (mm)}}{(\text{original width, mm})(\text{original thickness, mm})}$$

$$\text{Elongation at break (\%)} = \frac{\text{Increasing in length at break point (mm)x 100}}{\text{Original length(mm)}}$$

2.3.6.6 Fourier-transform infrared (FTIR) spectroscopy

The infrared spectra of the films were obtained within 4000-400 cm^{-1} range, using the spectrum one FT-IR spectrometer (Perkin Elmer, USA) with ATR accessory with ZnSe crystal.

2.3.6.7 X-ray diffraction

The physical state of the chitosan films was assessed by XRD studies. X-ray diffraction spectra of chitosan films were obtained at room temperature by using a X-ray diffractometer (X′pert MPD, Philips, Netherlands), with Co as an anode material and graphite monochromator, operated at a voltage of 40 kV. The sample were analyzed in the 2θ angle rang 5-90° and the process parameters were set as a step size of 0.05° (θ) and scan step time of 1.0 sec.

2.3.7 Acute dermal irritation test (OECD, 2002)

The test of acute dermal irritation/corrosion was done by Thailand institute of scientific and technological research using Albino rabbits of New Zealand white hybrid strain and dermal irritation scoring according to the test guideline (TG) no. 404 of the OECD guidelines for testing of chemical (2002). Three rabbits (three healthy adult albino rabbits of New Zealand white hybrid strain) were employed and acclimatized to the laboratory environment for one week. One day before experimentation, an area of skin approximately 10 cm x 10 cm on the dorso-lumbar region of each rabbit was clipped free of hairs. Two areas of the shaven skin approximately 2.5 cm x 2.5 cm were selected. The weight 0.5g of blank chitosan films cross-link with dP-ENR-25 or dP-ENR-50 were introduced onto a 2.5 cm x 2.5 cm gauze patch, which was served as a treated patch while 0.5 mL of distilled water on another patch was served as a control patch. Both patches were applied to the selected skin sites on each rabbit. The patches were then secured to the skin by transpore adhesive tape. The entire trunk of the rabbit was wrapped with elastic cloth to avoid dislocation of the patches for 4 h. At the end of the exposure period, all patches were removed and gently wiped the treated skin with moistened cotton wool to remove any residual test material. The animals were assessed for the degree of erythema and oedema evidence on each site at 1hr, 24, 48 and 72 h. After removal of the patched. Further observation would be needed, as necessary, to establish the reversibility if the irritation sign(s) still existed, but would not exceed 14 days after application. In addition to the observation of irritation, any lesions and other toxic effects were recorded. The skin reactions were independently scored by two inspectors using the numerical scoring system as follows:

Erythema and eschar formation:	**score**
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (Injuries in depth)	4

Oedema formation	**score**
No oedema	0
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well- defined by definite raising)	2
Moderate oedema (raised approximately 1 mm.)	3
Severe oedema (raised more than 1 mm. and extending Beyond the area of exposure)	4

2.4 Statistical analysis

Results are expressed as the mean$\pm$S.D. of at least three experiments. Analysis of variance (ANOVA) was used to test the statistical significance of differences among groups. The SPSS software package (SPSS 17.0 for Windows, SPSS Inc., Chicago, IL, USA). Comparison of means was performed by Duncan's test with confidence level as $P < 0.05$.

2.5 Result and discussion

2.5.1 Purification and characterization of epoxidized natural rubbers

2.5.1.1 Extraction of proteins from ENR

The allergy caused by rubber latex products has become a serious problem recently. Since, rubber latex from field collection contains many natural substances including 5% of non-rubbers components such as protein, lipid and sugar, with water accounting for the remaining 59% and 33% polyisoprene rubber. Even though, the natural rubber latex that will be used as starting material for ENR preparation is going to pass many step of preparation process. The proteins from natural rubber latex may still remain in the ENR product. So, if the ENR is continued to be utilized in chitosan film preparation, the remaining proteins will be existed in the resulting chitosan films and may not suitable for skin application. Therefore, the proteins remaining should be extracted from the ENR. The protein was easily removed by extraction procedure. The amount of extracted protein was assayed by Lowry's method and calculated by comparison with standard bovine serum albumin (BSA) (Figure 16). Amount of protein in ENR-25 and ENR-50 before extraction were 274$\pm$34.6 and 677$\pm$80.8 (μg/g), respectively. Amount of protein after purification process of in ENR-25 and ENR-50 are shown in Table 4 showed ability of extraction process.

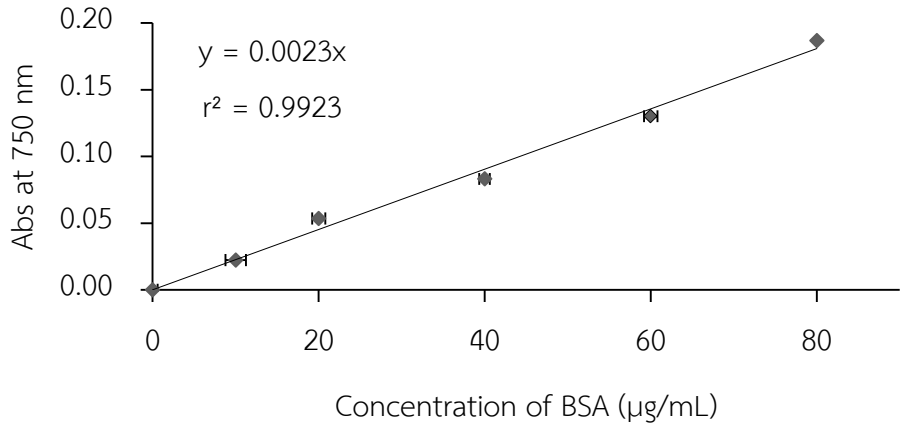


Figure 16. Standard curve of BSA determined by UV-Vis spectroscopy at 750 nm.

Table 4 Amount of protein before and after purification process of ENR.

Sample	Amount of protein determined by Lowry's method (µg/g)		
	Before	1st purification	2nd purification
ENR-25	274±34.6	121±25.6	ND*
ENR-50	677±80.8	80±16.6	ND*

* No detection.

2.5.1.2 Characterizations of deproteinated epoxidized natural rubbers

Table 5 shows number average molecular weight, ($\overline{M_n}$), weight average molecular weight, ($\overline{M_w}$) and polydispersity index (PI) of deproteinated ENR-25 (dP-ENR-25) and deproteinated ENR-50 (dP-ENR-50), respectively. The result indicated that both dP-ENRs have the molecular weights both number average molecular weight ($\overline{M_n}$) and weight average molecular weight, ($\overline{M_w}$) in the same range with high polydispersity index. Since, we would like to know whether the de-proteination process will effect the % epoxidation on the ENR, ^{1}H-NMR will be further utilized for % epoxidation of both dP-ENRs.

Table 5 number average molecular weight ($\overline{M_n}$), weight average molecular weight ($\overline{M_w}$) and polydispersity index (PI) of ENR-25 and ENR-50 after de-proteination process.

Sample	$\overline{M_w}$ (g/mol)	$\overline{M_n}$ (g/mol)	PI
dP-ENR-25	475,310	168,970	2.84
dP-ENR-50	441,520	151,330	2.93

^{1}H-NMR spectra of dP-ENR-25 and dP-ENR-50 are displayed in Figure 17. The proton resonances of dP-ENR-25 and dP-ENR-50 are assigned to at 1.29 ppm are methyl protons bonded to the epoxidized isoprene unit (a) (–CH_2–C**H_3**COCH–CH_2–); at 1.55 ppm are methylene protons bonded to the epoxidized isoprene unit (b) (–C**H_2**–CH_3COCH–CH_2–), at 1.67 ppm are methyl protons bonded to the isoprene unit (c) (CH_2–C**H_3**C=CH–CH_2–), at 2.04 ppm are methylene protons bonded to the isoprene unit (d) (-C**H_2**–CH_3COCH–CH_2–) overlapped with the methylene protons bonded to the epoxidized isoprene unit (e) (–CH_2–CH_3COCH–C**H_2**–); at 2.15 ppm are methylene protons bonded to the isoprene unit (f) (–CH_2–CH_3COCH–C**H_2**–); at 2.70 ppm are methine proton bonded to the epoxidized isoprene unit (g) (–CH_2–CH_3COC**H**–CH_2–); at 5.10 ppm is a methine proton bonded to the isoprene unit (h) (–CH_2–CH_3C=C**H**–CH_2–). The presence of residual amount of chloroform can be observed at 7.5 ppm. The epoxy content of dP-ENR-25 and dP-ENR-50 were estimated from the intensity ratio of the signals at 2.7 and 5.1 ppm, calculated by the following equation: (Hamzah *et al.*, 2012, Mas Haris and Raju, 2014) and the results are summarized in Table 6.

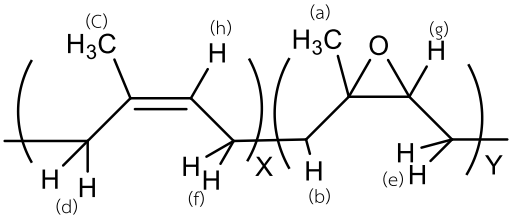
$$\%\text{Epoxidation} = \frac{I_{2.70}}{I_{2.70} + I_{5.10}} \text{x } 100$$

Where $I_{2.70}$ and $I_{5.10}$ are integrated area of peaks at chemical shifts 2.70 and 5.14 ppm, respectively.

Table 6 %Epoxidation before and after de-proteination process determined by ^{1}H-NMR

Sample	Before de-proteination	After de-proteination
dP-ENR-25	30.08	29.08
dP-ENR-50	53.91	51.80

*dP-ENR = deproteinated ENR



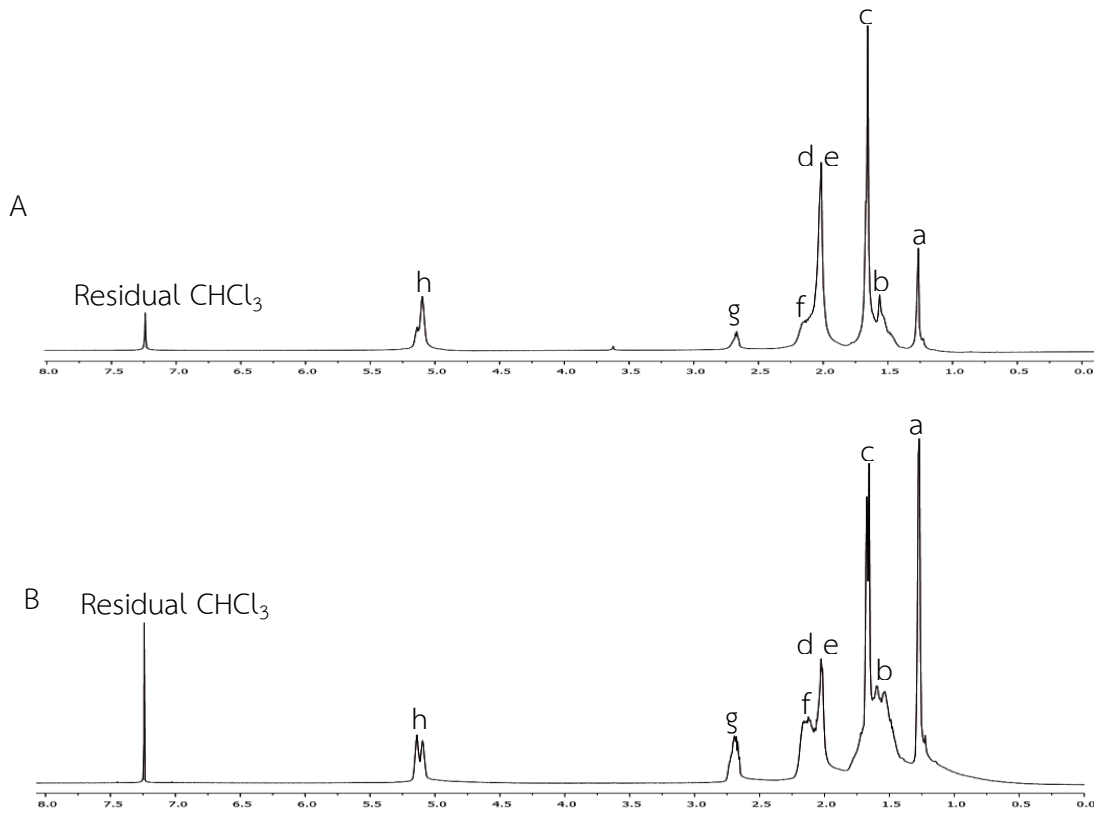


Figure 17 ^{1}H NMR (500 MHz) spectra for dP-ENR-25 (A) and dP-ENR-50 (B) in $CDCl_3$

2.5.2 Optimization for a suitable ratio between type of solvent and epoxidized natural rubbers for cross-linking reaction.

The cross-linked chitosan preparation has to be done as the solution, therefore the suitable solvent is required to afford the best condition. The solvent included dichloromethane, dimethylsulphoxide, tetrahydrofuran, and toluene were selected for this investigation and the result are summarized in Table 7.

Table 7 The solubility of dP-ENR in the selected sovents.

Solvent (100 mL)	dP-ENR-25 (5g)	dP-ENR-50 (5g)
Dichloromethane	Swell	Swell
Dimethylsulphoxide	Insoluble	Insoluble
Tetrahydrofuran	Soluble	Soluble
Toluene	Soluble	Soluble

The both dP-ENRs were very well dissolved in tetrahydrofuran and toluene after 48 h stirring. However, tetrahydrofuran will be selected to be used as a solvent for preparation dP-ENR solution, since it is miscible with water. According to the preparation of chitosan film, chitosan will be dissolve in 1% acetic acid solution, therefore, if toluene is used in the mixing process, phase separation will occur.

2.5.3 Optimization for a suitable concentration of dP-ENR in tetrahydrofuran for cross-linking reaction.

Since during film preparation process dP-ENR will be used in a solution form, therefore the solubility property of dP-ENR in THF was investigated. Amount of dP-ENRs were varied from 0.1-10% in THF and the solubility property were observed and displayed in Table 8. The result showed that solution of dP-ENRs can be obtained when using dP-ENRs in a range of 0.1-5% w/v. Both dP-ENRs were not completely soluble in THF when 10% of both dP-ENRs were used.

Table 8 Solubility property different concentrations of dP-ENR in THF.

Concentration of dP-ENR in tetrahydrofuran	dP-ENR-25	dP-ENR-50
0.1%	Soluble	Soluble
0.2%	Soluble	Soluble
0.5%	Soluble	Soluble
1.0%	Soluble	Soluble
2.0%	Soluble	Soluble
3.0%	Soluble	Soluble
4.0%	Soluble	Soluble
5.0%	Soluble	Soluble
10%	Insoluble	Insoluble

2.5.4 Optimization for a suitable protocol for chitosan films preparation

The preparation process for chitosan films were optimized by varying the factors that may affect the preparation process, including polymer concentrations, cross-linker concentrations and volumes of cross-linker.

2.5.4.1 Investigation for a suitable concentration of dP-ENRs.

Chitosan cross-linked films with dP-ENRs were prepared by casting method. Therefore, the chitosan solution that suitable for this method should give clear solution without any aggregation. In the investigation, 1.5 g of chitosan was dissolved in 100 mL 1% acetic acid and 1 mL of each concentration of dP-ENR in THF was added and stirred at RT for 24 h. The result showed that clear solution can be obtained when using 1 mL dP-ENR-25 in THF at the concentration from 0.1% to 40% w/v. Aggregates were observed when 5% w/v of dP-ENR-25 (1 mL) was added to the chitosan solutions. Clear solutions were obtained when dP-ENR-50 solutions at concentration from 0.1-1.0% w/v was added to the chitosan solution after stirring for 24 h. Aggregates were found in the mixture of the chitosan solution with dP-ENR-50 solutions at concentration 2.0-5.0% w/v. From these results, suitable concentrations of dP-ENR-25 and dP-ENR-50 which will be used in the films preparation process are 4% and 1% w/v in THF, respectively.

Table 9 Optimization of a suitable concentration of epoxidized natural rubber for preparation of chitosan films.

Concentration of dP-ENR-25 (w/v) in THF	Observation	Concentration of dP-ENR-50 (w/v) in THF	Observation
0.1%	Soluble	0.1%	Soluble
0.25%	Soluble	0.25%	Soluble
0.5%	Soluble	0.5%	Soluble
1.0%	Soluble	1.0%	Soluble
2.0%	Soluble	2.0%	Aggregate
3.0%	Soluble	3.0%	Aggregate
4.0%	Soluble	4.0%	Aggregate
5.0%	Aggregate	5.0%	Aggregate

2.5.4.2 Optimization for a suitable volume of epoxidized natural rubbers for chitosan film preparation.

Next investigation was to determine the suitable volumes of dP-ENR-25 and dP-ENR-50 in film preparation that will be added to the 1.5% chitosan solution. The experiments were performed using 1.50 g chitosan in 100 mL 1.0% acetic acid, the solution of 4.0% dP-ENR-25 and 1.0% dP-ENR-25, 0.25-3.0 mL were added. An optimal volume of 4% dP-ENR-25 and 1% dP-ENR-50 was selected from the maximum volume that gave clear solution after adding to the 1.5% w/v chitosan solution. The result found that when increasing volumes of both dP-ENRs from 0.25-1.00 mL, clear solutions were observed (Table 10). However, aggregates were noticed when using both dP-ENR solutions of any volumes above 1 mL (1.25-3.00 mL). Therefore, in the chitosan films preparation, 1 mL of 4% w/v dP-ENR-25 and 1% w/v dP-ENR-50 were selected.

Table 10 Optimization for a suitable volume of ENR in THF for chitosan films preparation.

4.0 % w/v dP-ENR-25 (mL)	Observation	1.0 % w/v dP-ENR-50 (mL)	Observation
0.25	Soluble	0.25	Soluble
0.50	Soluble	0.50	Soluble
0.75	Soluble	0.75	Soluble
1.00	Soluble	1.00	Soluble
1.25	Aggregate	1.25	Aggregate
1.50	Aggregate	1.50	Aggregate
2.00	Aggregate	2.00	Aggregate
2.50	Aggregate	2.50	Aggregate
3.00	Aggregate	3.00	Aggregate

2.5.4.3 Optimization of chitosan concentration.

Then the concentration of chitosan solution for film preparation was investigated. The reaction was performed by using 100 mL of chitosan solution with varying concentrations at 1.00, 1.25, 1.50, 2.00 and 2.25% w/v in 1% acetic acid and mixed with 1 mL of 4% w/v dP-ENR-25 or 1.0% w/v dP-ENR-50. The optimal concentration of chitosan solution for films preparation were optimized on the basis of clear solution after the reaction mixture was stirred at 24 h. The results in Table 11 indicated that clear solutions were obtained when using 100 mL of chitosan solution at concentrations at 1.00, 1.25 and 1.50% w/v after mixing with 1 mL of both dP-ENRs. Aggregates were observed when increasing concentrations of chitosan solutions from 1.75-2.25% w/v. Hence, in the film preparation process, 100 mL of 1.5% w/v of chitosan solution was chosen for mixing with 1 mL of 4% dP-ENR-25 or 1 mL of 1% dP-ENR-50 and stirred for 24 h before film casting.

Table 11 Optimization for a suitable concentration of chitosan solution (100 mL) for chitosan films.

Concentrations of chitosan solutions (%w/v)	Observation after mixing with 1 mL, 4% dP-ENR-25	Observation after mixing with 1 mL, 4% dP-ENR-50
1.00	Soluble	Soluble
1.25	Soluble	Soluble
1.50	Soluble	Soluble
1.75	Aggregate	Aggregate
2.00	Aggregate	Aggregate
2.25	Aggregate	Aggregate

2.6.4.4 The optimum formulation for blank chitosan films preparation

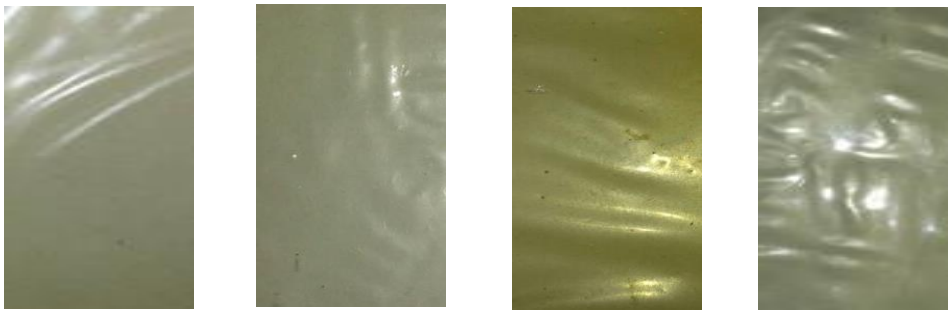
From the previous investigation, the optimum conditions used for blank chitosan films preparation were 1.5 g chitosan in 1.0% acetic acid solution 100 mL and mix with, 1.0 mL of 1.0% dP-ENR-50 or 1.0 mL of 4.0% dP-ENR-25 (Table 12). In film preparation process 100 mL of 1.5% w/v of chitosan solution was mixed with 1 mL of 4% w/v of dP-ENR-25 or 1 mL of 1% w/v of dP-ENR-50 and stirred at RT for 24 h before casting. The films were obtained after drying in an oven at 40 ^{o}C and used for physico-chemical properties determination.

Table 12 The formulation used for blank chitosan films preparation.

Composition	Volume
1.5% w/v of chitosan in 1% acetic acid	100 mL
4% w/v dP-ENR-25 in THF or 1% w/v dP-ENR-50 in THF	1 mL

2.5.5 Physicochemical characterization

Chitosan films were successfully obtained from the reaction between chitosan with, dP-ENR-25 or dP-ENR-50 and EGDE. The appearances of different films are shown in Fig. 18. The chitosan films of all formulations were obtained as slightly yellow and transparent films with some wrinkle resulted from the drying process.



(a) (b) (b) (d)

Figure 18. Pictures of chitosan films, (a) Chitosan film without cross-linking agent (CH); (b) chitosan film cross-linked with dP-ENR-25 (CH-dP-ENR-25); (c) chitosan film cross-linked with dP-ENR-50 (CH-dP-ENR-50); (d) chitosan film cross-linked with EGDE (CH-EGDE).

2.6.5.1 Microstructural characterization

SEM observation of the chitosan films from different formulations are shown in Fig. 19. The figures displayed surface and cross-sections of each film. Chitosan films without the cross-linking agent (CH, Fig. 19a) showed a smoother surface than those obtained from the formulations with cross-linking agents (Figs. 19c, e, g). With the additional cross-linking agents in the film preparation process, the surface structure changed markedly. The surface of films with dP-ENR displayed some irregular holes with fine spherical particles. This observation was similar to that previously reported in the

utilization of PEG blended with chitosan (He *et al.*, 2009). In contrast, the surface of the film obtained using EGDE as a cross-linking agent (Fig 19g), showed some irregular aggregates. The cross-section morphologies of the obtained films were determined and these micrographs are illustrated in Fig.19b, d, f, and h. The cross-section morphologies of the CH-dP-ENRs (Fig.19d and f) and CH-EGDE (Fig.19h) films were denser than the CH-film (Fig.19b). Pores were observed in the CH-film and CH-dP-ENR-50 films, whereas, the CH-dP-ENR-25 and CH-dP-ENR-50 films were non-porous. A number of aggregates were detected inside the cross-sections of the CH-EGDE film, and this could be due to the self-aggregation of chitosan with a high cross-linking reaction that resulted in self-gathering during solution volatilization.

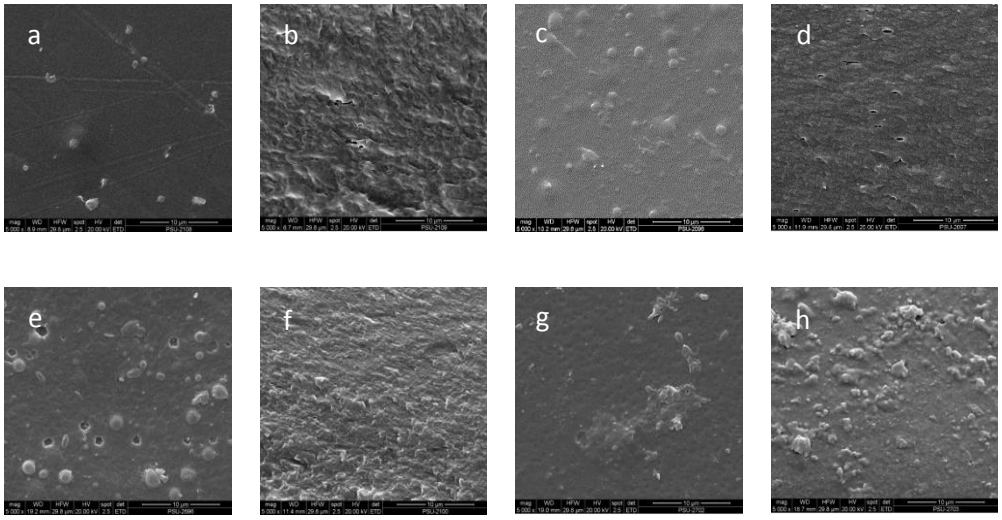


Figure 19. SEM micrographs of the obtained chitosan films at 5000x magnification (a, c, e , g) surface appearance ; (b, d, f, h) cross sections; (a, b) CH-film ; (c, d) CH-dP-ENR-50-films; (e, f) CH-dP-ENR-25-films; (g, h) CH-EGDE-film.

2.5.5.2 Film swelling property

The equilibrium water contents of the chitosan films cross linked by dP-ENR or EGDE and chitosan films without cross-linking in phosphate buffer (pH 7.4) are shown in Figure 20. It was found that the cross-linked films showed higher degrees of swelling compared to non-cross-linked chitosan film, indicating higher hydrophilicity. The degree of swelling for the cross-linked films were found to be in the range of 151-181%, while the degree of swelling for non-cross-linked chitosan films was 140% respectively. The higher swelling of the cross-linked film as compared with non-cross-linked chitosan film may be due to the fact that the epoxy group of ENR might have hydrogen bonding interaction with water in solution. Moreover, the films cross-linked showed increased swelling which could be due to the increase in phase separation between chitosan and dP-ENR molecules, resulting in increased free volume in the film matrix. The increase in free volume of the polymer system enhanced the diffusion of small molecules in the polymer matrix and could provide the swelling increase as a function of free-volume hole size (Wang *et al.*, 2003). In addition, hydroxyl groups presented in the chitosan molecules and resulted from ring opening reaction of ENR are hydrophilic groups, which might promote water transfer in the matrix (Sun *et al.*, 2014)**.** Hermans *et al* (2014) reported that the degree of swelling depends on the resistance of the matrix structure to the movement of water molecules. A high number and strength of hydrogen bonds between polymer chains result in the formation of a strong network structure that resists the rapid penetration of water**.** The high swelling capacity of chitosan-based inserts is attributed to the hydrophilic nature of chitosan. Hydroxyl and amino groups present in its structure have the ability to interact with water molecules (Hermans *et al*., 2014). Lavorgna *et al* (2010) reported that the water uptake of chitosan films containing glycerol was approximately 10 times lower compared to non-plasticized films. Glycerol molecules displaced the bound acetic acid in the formation of hydrogen bonds with the amine groups of chitosan, resulting in an equilibrium swelling index value of approximately 1.5

(Lavorgna *et al.*, 2010). In the present study, the cross-linked films exhibited higher water uptake, and reaching a swelling index of more than 1.51.

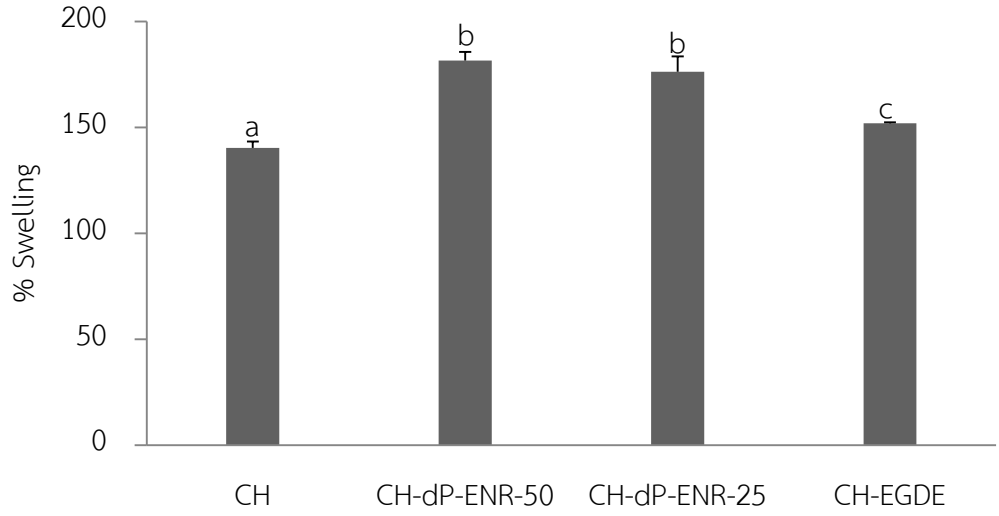


Figure 20. Swelling behavior for blank chitosan films, film without cross-linked agent (CH); chitosan cross-linked with dP-ENR-50 (CH-dP-ENR-50); chitosan cross-linked with dP-ENR-25 (CH-dP-ENR-25); chitosan cross-linked with EGDE (CH-EGDE).

Different superscripts letters (a–d) within the same column indicate significant differences between formulations ($P < 0.05$). ANOVA was used to test the statistical significance of differences among groups.

2.5.5.3 Moisture uptake capacity

The moisture uptake capacity represented the maximum amount of water the films could absorb and this should relate to their swelling and mechanical properties. The CH-dP-ENR-25, CH-dP-ENR-50 and CH-EGDE films showed a significant increase in their moisture uptake capacity as compared with the CH-film ($P < 0.05$, Fig. 21). This could be because of the cross-linked chitosan films had expanded to form an internal three dimensional structure that held more water molecules inside the space than the non-

cross-linked chitosan film. The absorbed moisture may act as plasticizer and thus the films with the higher moisture uptake capacity would show more tensile strength as compared to the non-cross-linked film.

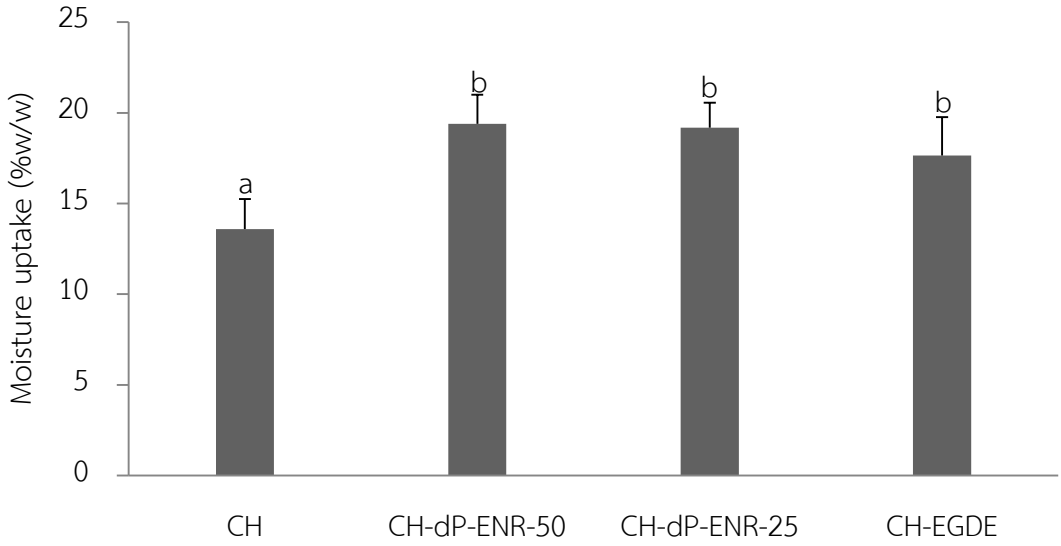


Figure 21. Moisture uptake for blank films, chitosan film without cross-linking agent (CH); chitosan cross-linked with dP-ENR-50 (CH-dP-ENR-50); chitosan cross-linked with dP-ENR-25 (CH-dP-ENR-25); chitosan cross-linked with EGDE (CH-EGDE).

Different superscripts letters (a–b) within the same column indicate significant differences between formulations ($P < 0.05$). ANOVA was used to test the statistical significance of differences among groups.

2.5.5.4 Mechanical properties

The mechanical properties of the chemically cross-linked chitosan films are shown in Figures 22-23 and compared with those of the non-crossed-linked chitosan film. The thickness of the films were varied from 31 to 32 μm, and these values will be used to calculate the tensile stress at break. The CH-dP-ENR films had a significantly higher tensile strength than the CH-film and CH-EGDE films ($P < 0.05$), due to a strong covalent

interaction between the chitosan chain with the ENR, leading to a strong 3D network films (Hashemi Doulabi *et al.*, 2013, Ozcelik *et al.*, 2013). The EBA values of the CH-dP-ENR and the CH-EGDE films also increased significantly when compared with the CH-film that may resulted from their stronger 3D network formation. Since ENR is known to have a high elastic characteristic (Zurina *et al.*, 2008), it's inter-connection between the chitosan chains would support the improvement of the EBA of the cross-linked films. The tensile strength values (TS) of the non-cross-linked chitosan film and cross-linked chitosan film with EGDE, dP-ENR-25, dP-ENR-50 were 48.07, 75.98, 94.20 and 137.01, respectively. This indicated that using ENR as crosslinking agent could significantly elevate the elasticity property of the chitosan films. The improvement was found to be much better than in the non-cross -linked and the EGDE cross-linked chitosan films. The results demonstrated that utilizing dP-ENR-50 as a cross linking agent could improve the film's elasticity by greater than 3- and 2-times that of the non-cross-linked and in EGDE cross-linked chitosan films, respectively. It was obvious that the chitosan cross-linked with ENR could enhance film flexibility better than EGDE and the chitosan without cross-linking agent. High flexible films are suitable for bio-medical application as they can resist the body movement at the applied site. Soheilmoghaddam *et al* (2013) reported cellulose blend with epoxidized natural rubber for film preparation and investigated for the mechanical properties of dry and wet film samples. The results showed that the regenerated cellulose exhibited the highest tensile strength as well as Young's both in dry and wet state. This may be due to the incorporation of weaker ENR component in to the regenerated cellulose, therefore, ENR can greatly enhance the flexibility of dry and wet films.

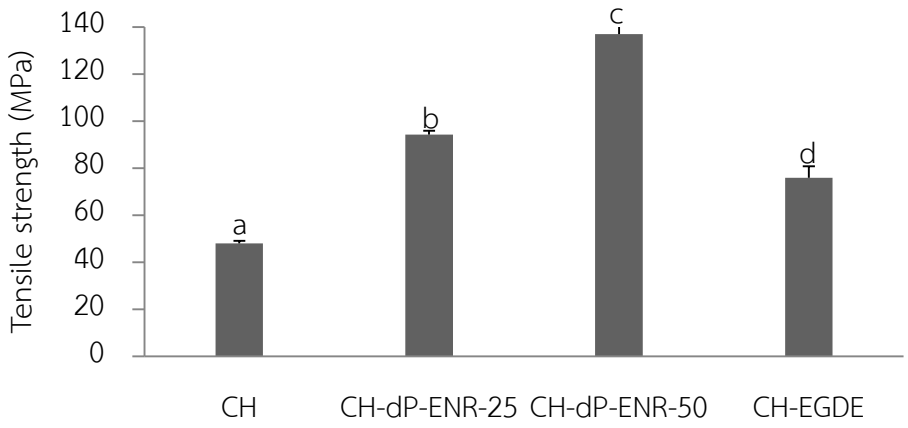


Figure 22. Tensile strength for blank film; chitosan film without cross-linking agent (CH); chitosan cross-linked with dP-ENR-50 (CH-dP-ENR-50); chitosan cross-linked with dP-ENR-25 (CH-dP-ENR-25); chitosan cross-linked with EGDE (CH-EGDE).

Different superscripts letters (a–d) within the same column indicate significant differences between formulations ($P < 0.05$). ANOVA was used to test the statistical significance of differences among groups.

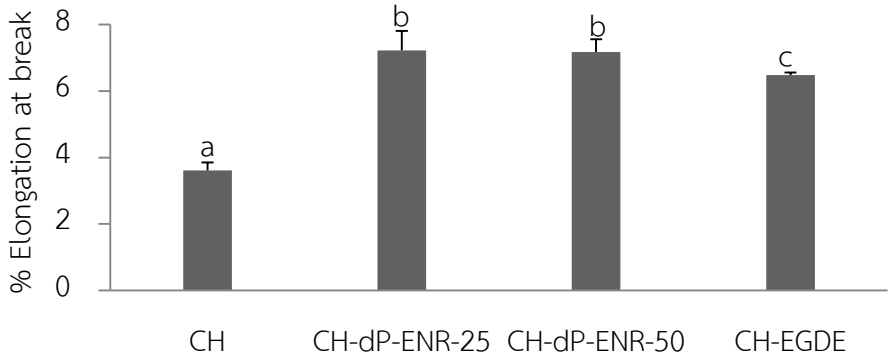


Figure 23. Elongation at break of blank films, chitosan without cross-linking agent (CH); chitosan cross-linked with dP-ENR-50 (CH-dP-ENR-50); chitosan cross-linked with dP-ENR-25 (CH-dP-ENR-25); chitosan cross-linked with EGDE (CH-EGDE).

Different superscripts letters (a–c) within the same column indicate significant differences between formulations ($P < 0.05$). ANOVA was used to test the statistical significance of differences among groups.

2.5.5.5 Fourier-transform infrared (FTIR) spectroscopy

The ATR-FTIR spectra of the ENR having 25 and 50 mole% of epoxide content (Figs. 24a, b) showed absorption peaks at 875 and 1251 cm^{-1} corresponded to the asymmetrical and symmetrical ring stretching of the epoxide group. The absorption peaks at 2963, 2926, 2860 cm^{-1} (C-H stretching), 1664 cm^{-1} (C=C stretching), 1451 cm^{-1} (-CH_2-deformation), 1378 cm^{-1} (methyl C-H deformation) and 838 cm^{-1} (C–H deformation of cis -C=C–H) were detected similar to that previously reports (Thongnuanchan *et al.*, 2007, Amornchaiyapitak *et al.*, 2008, Boonsong *et al.*, 2008). Chitosan blank films without cross-linking agent (CH, Fig. 24d) showed a broad ¯OH stretching vibration band of chitosan at 3000-3750 cm^{-1} that overlapped to the stretching vibration of -N-H. The absorption bands at 2921 and 2870 cm^{-1} were assigned to asymmetrical and symmetrical –CH_2 and CH_3 groups, respectively. Absorption in the range of 1680 – 1480 cm^{-1} was related to the vibrations of the carbonyl moieties of the amide (ν = 1638 cm^{-1}) and to the protonated amine groups (ν = 1560 cm^{-1}) (Pawlak and Mucha, 2004, Buraidah and Arof, 2011). The bands observed at 1420, 1380 and 1334 cm^{-1} were attributed to the C-N stretching vibration, and the bands at 1256 cm^{-1} corresponded to the –OH bending vibration. The C-O stretching vibration for a secondary alcohol was observed at 1150 cm^{-1}, while a C-O stretching vibration for primary alcohol was observed at 1065 and 1028 cm^{-1}. The bands at 894 belonged to a C-H out-of-plane bending vibration and the bands observed at 604 cm^{-1} represented a –NH_2 wagging vibration (Martins *et al.*, 2012). Meanwhile, the characteristic absorption bands of chitosan cross-linked with dP-ENR and EGDE are displayed in Figs. 24e-g. The intensity of the –NH bending vibration band at 1636 cm^{-1} of chitosan decreased significantly in the obtained product after a cross-linking reaction and shifted slightly to a lower frequency of the second primary amine band to 1539 cm^{-1}. The films cross-linked with dP-ENR decreased the intensity of the C-N vibration, particularly for the C-N vibration at 1380 cm^{-1} of the natural chitosan. The C-N vibration bands were shifted to 1410, 1377 and 1319 cm^{-1}. The physical mixture between chitosan and ENR (Fig 24c) displays FT-IR absorption bands that were characteristics of both the chitosan and

the epoxide of ENR at 875, 1251 cm^{-1}, indicated that no interaction between the ENR and chitosan (Kamari *et al.*, 2011). However, the characteristic absorption bands of the epoxide decreased after the cross-linking reaction in the CH-dP-ENR films, indicated the chemical interaction between the NH_2 of chitosan with the epoxide groups of ENR and EGDE had occurred. This was similar to previous observations (Riyajan and Sukhlaaied, 2013, Mas Haris and Raju, 2014).

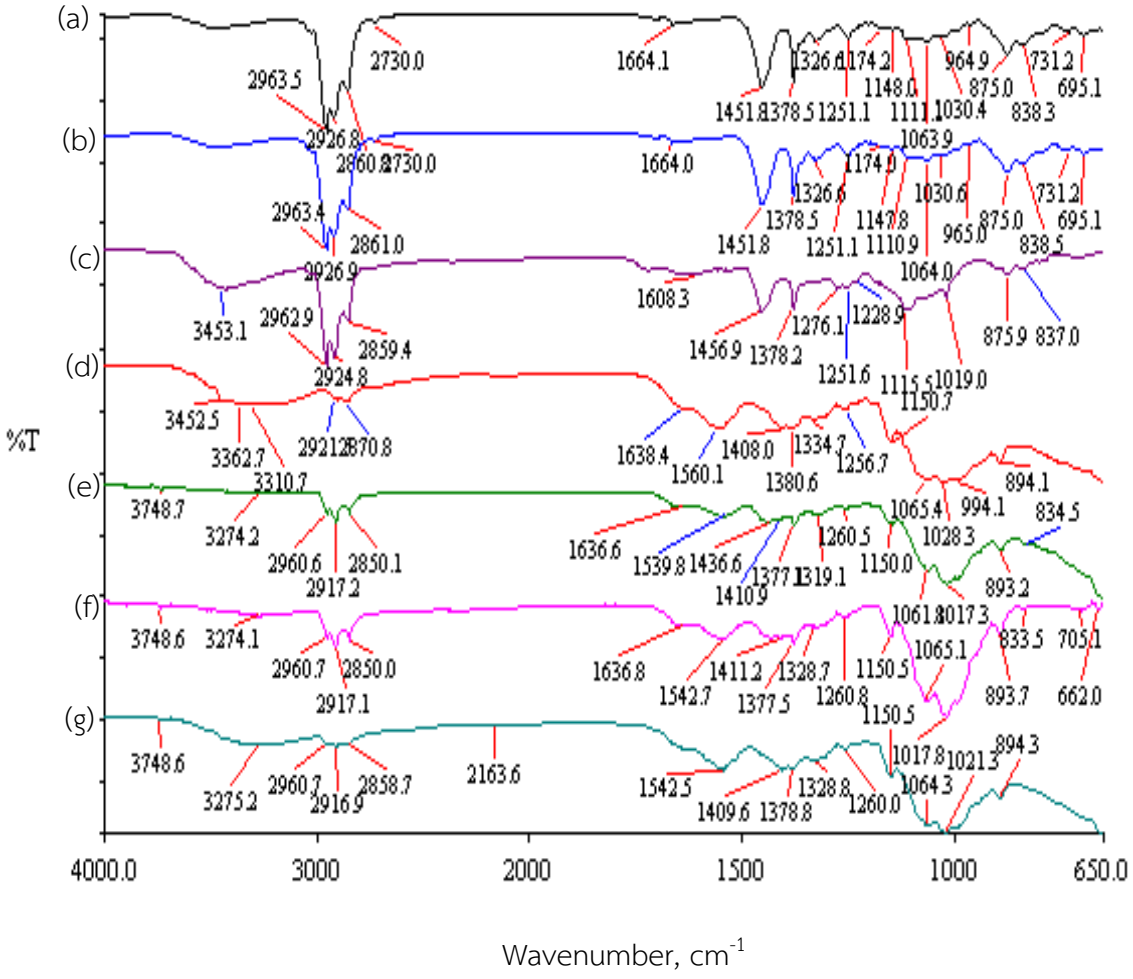


Figure 24. FT-IR spectra of blank chitosan films, (a) dP-ENR-25; (b) dP-ENR-50; (c) Physical mix between chitosan and dP-ENR-25; (d) CH; (e) CH-dP-ENR-25; (f) CH-dP-ENR-50; (g) CH-EGDE.

2.5.5.6 X-ray diffraction patterns of the films

To study the effect of cross-linking on the films crystallinity property, X-ray diffraction was employed and the X-ray diffraction patterns are presented in Fig. 25. A chitosan film without cross-linker exhibited four broad peaks of 2θ at around 8.6°, 11.6°, 18.4° and 23.2° in the diffraction pattern (Fig. 25d), and is due to an existence of amorphous and crystalline regions, respectively. Upon increasing the cross-linking density (Fig. 25a-c), all sharp peaks in cross-linked chitosan films decreased gradually (Leceta *et al.*, 2013). It may be due to decreased crystalline domains in the membrane matrix because the functional groups ($-NH_2$ and –OH) present in chitosan membrane underwent significant change after cross-linking (Choudhari *et al.*, 2007). The results indicated that the cross-linked chitosan had a less crystalline structure as compared to non-cross-linked chitosan film (Leceta *et al.*, 2014). The CH-film shows that chitosan is a semicrystalline polymer, which exhibit two main reflection peaks at 2θ = 11.6 and 18.4 (Dehnad *et al.*, 2014, Inta *et al.*, 2014, Lopez *et al.*, 2014). The strong reflection appeared at 2θ of 11.6° was assigned to hydrated crystal, and the reflections fell at 2θ of 18.4° and 23.2° were attributed to both hydrated crystal and amorphous structures. Moreover, the high crystallinity of chitosan is also caused by inter- and intramolecular hydrogen bonds.

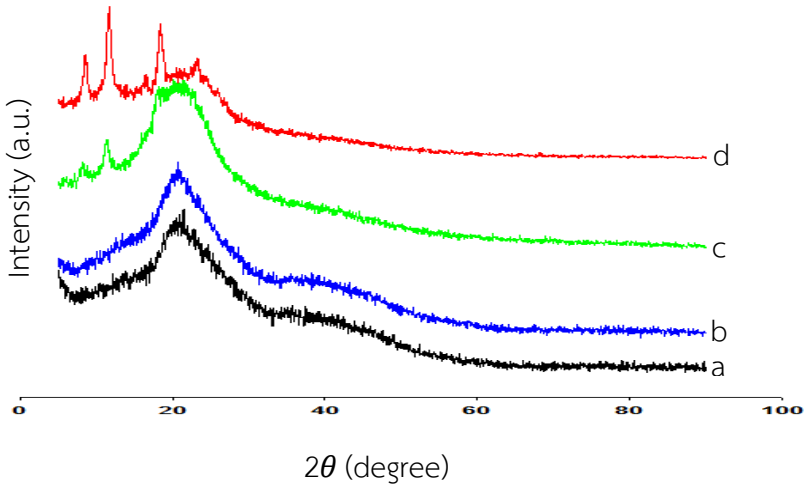


Figure 25. X-ray spectra of blank chitosan films, (a) CH-dP-ENR-25; (b) CH-dP-ENR-50; (c) CH-EGDE; (d) CH.

2.5.6 Acute dermal irritation test

The acute dermal test of the chitosan films cross-linked with dP-ENR-25 and -50 was performed by applying the films on the rabbit's skin, compare with water as a control. After removal of the patches, the treated skin of each rabbit was examined for skin reactions at 1, 24, 48 and 72 h. The scores of dermal reactions of the rabbits treated with distilled water (control area) and chitosan films cross-linked with dP-ENR-25 or dP-ENR-50 (treated area) shown in Tables 13, 14 and 15, respectively. The result demonstrated that both chitosan films obtained from the cross-linked reaction with dP-ENR-25 and dP-ENR-50 did not show any type of dermal irritation even after application on the rabbit's skin for 72 h. The result from the skin irritation test indicated that the resulting chitosan films are safe to be used for skin drug delivery.

Table 13 The score of dermal reaction of the control area on the skin of the rabbits treated with distilled water

	Scoring of dermal reaction							
Application time (h)	1		24		48		72	
Rabbit No.	Erythema	Oedema	Erythema	Oedema	Erythema	Oedema	Erythema	Oedema
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0

Table 14 The score of dermal reaction of the treated area on the skin of the rabbits treated with chitosan films cross-linked with dP-ENR-25.

	Scoring of dermal reaction							
Application time (h)	1		24		48		72	
Rabbit No.	Erythema	Oedema	Erythema	Oedema	Erythema	Oedema	Erythema	Oedema
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0

Table 15 The score of dermal reaction of the treated area on the skin of the rabbits treated with chitosan films cross-linked with dP-ENR-50.

	Scoring of dermal reaction							
Application time (h)	1		24		48		72	
Rabbit No.	Erythema	Oedema	Erythema	Oedema	Erythema	Oedema	Erythema	Oedema
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0

2.5.7 The cross-linking reaction mechanism

The plausible cross-linking reaction mechanism between chitosan, a major film component and ENR as cross-linking agent are proposed in Fig. 26. Since, chitosan was previously dissolved in acidic medium, it is therefore known that it will form a polyelectrolyte due to the protonation of the $-NH_2$ groups (Rinaudo *et al.*, 1999). The

epoxidized units of ENR would likely be also protonated and readily for ring opening to form carbocations at the more substituted carbons (Gelling, 1987). In this case the unhindered hydroxyl groups of chitosan chain at C-6 positions are much more nucleophilicity than quaternary ammonium groups, the formers will undergo attack at the carbocations of the ENR resulting in cross-linked products. This possible cross-linking mechanism are proposed base on the previous report of the chitosan modification by cross-linking reaction with EGDE (Li and Bai, 2004) in which the cross-linking reaction took place at the hydroxyl groups at C-6 positions in chitosan whereas the amine groups were unreactive. Moreover, from FT-IR spectra of both CH-dP-ENR films showed that the band for –OH and –NH stretching vibration around the wavenumber 3439 cm^{-1} were significantly reduced when compared to that in non-cross-linked chitosan film and in physical mixture of chitosan and ENR. The results suggested that the cross-linking reaction occurred via acid catalyzed ring opening with the –OH of -CH_2-OH (major) and perhaps the –NH_2 of –C-NH_2 (minor) as nucleophiles. In contrast to the CH-EGDE film, the cross-linking reaction was reported to occur with –NH_2 of –C-NH_2 as can be seen in the FT-IR spectrum (Fig.24g) that the band for –OH stretching vibration at 3000 – 3750 3439 cm^{-1} were preserved and the bands 1420 and 1320 3439 cm^{-1}, relating to the amine groups were weakened after EGDE crosslink reaction (Li and Bai, 2004).

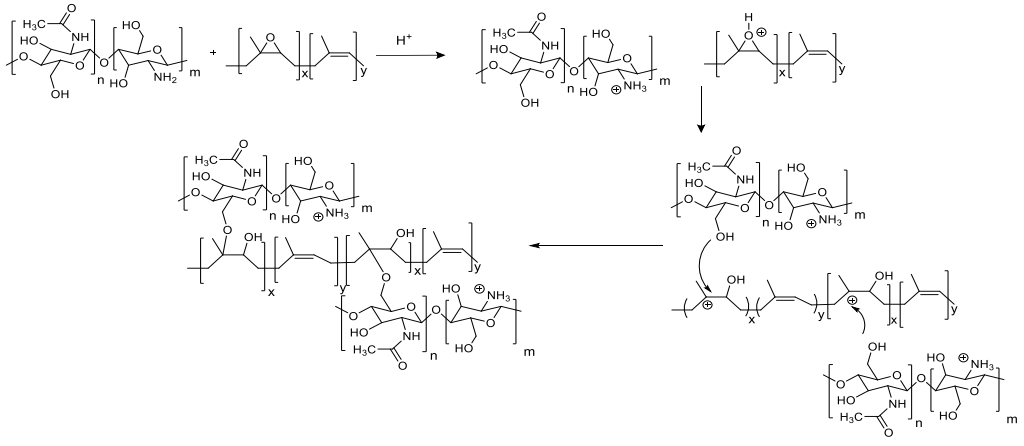


Figure 26. Schematic represent possible cross-linking mechanisms for the formation of CH-dP-ENR films.

2.6 Preparation of chitosan films cross-linked with dP-ENR containing diclofenac.

Diclofenac was selected as a model drug in chitosan films cross-linked with dP-ENRs for skin drug delivery as pain removal patch. Several factors were optimized to obtain proper formulations for chitosan films preparation as following.

2.6.1 Optimization of chitosan films cross-linked with dP-ENR containing diclofenac in chitosan films preparation

Chitosan films cross-linked with dP-ENR containing diclofenac were prepared by dissolving chitosan (1.50g) in 1.0% acetic acid solution (100 mL) at room temperature (30$\pm$1^{o}C) with magnetic stirrer for 24 h. 1.0% dP-ENR-50 or 4.0% dP-ENR-25 (1 mL) in tetrahydrofuran was added to the chitosan solution and stirred at room temperature (30$\pm$1^{o}C) for 24 h. Diclofenac at 1.00, 2.50, 5.00, 7.50, 10.00, 12.50, 15.00, 17.50, 20.00, 22.50, 25.00, 27.50, 30.00, 32.50 and 35.00 mg was separately dissolved in 5.0 mL methanol and mixed with 1.0 mL PEG-200 and was added to the chitosan solution and stirred at room temperature (30$\pm$1^{o}C) for another 24 h. The resulting mixtures were casted on a petri dish and spread manually to the outside borders. The films were dried in an oven at 40 ^{o}C for 48 h. The suitable formulation was selected form the formulation that gave transparent films and did not give any precipitation in the resulting films.

2.6.2 Optimization for amount of plasticizer (PEG-200) for films formulations.

PEG-200 was added to the formulation as a plasticizer, since the films without PEG-200 were less flexible and very bristle. The chitosan films with plasticizer were prepared

by dissolving chitosan (1.5 g) in 1.0% acetic acid solution (100 mL) at room temperature (30±1°C) with magnetic stirrer for 24 h. 1.0% dP-ENR-50 or 4.0% dP-ENR-25 (1 mL) in tetrahydrofuran was added to the chitosan solution and stirred at room temperature (30±1°C) for 24 h. Diclofenac, 20 mg dissolving in 5.0 mL methanol. PEG-200 in varying volumes at 0.25, 0.5, 0.75, 1.00, 1.25, 1.50 and 2.00 mL was added to the diclofenac solution before adding to the chitosan–dP-ENR solution and stirred at room temperature (30±1°C) for 24 h. The resulting mixtures were casted on a petri dish and spread manually to the outside borders. The films were dried in an oven at 40 °C for 48 h. The suitable formulation was selected form the formulation that gave clear transparent with good flexible films and did not give any precipitation in the resulting films.

2.6.3 Preparation process for chitosan films containing diclofenac

2.6.3.1 Chitosan film without cross-linker containing diclofenac

Chitosan films were prepared by dissolving chitosan (1.50 g) in 1.0% acetic acid solution (100 mL) at room temperature (30±1 °C) with magnetic stirrer for 24 h. Diclofenac (20 mg) was separately dissolved in 5 mL methanol and mixed with 1.0 mL PEG-200) was then added to the chitosan solution and stirred at room temperature (30±1 °C) for another 24 h. The solution was casted on a petri dish and spread manually to the outside borders. The films were dried in an oven at 40 °C for 48 h.

2.6.3.2 Chitosan films cross-linked with dP-ENRs containing diclofenac

Chitosan films cross-linked with dP-ENRs were prepared by dissolving chitosan (1.50 g) in 1% acetic acid solution (100 mL) at room temperature (30±1 °C) with magnetic stirrer for 24 h. Solution of 4.0% dP-ENR-25 or 1.0% dP-ENR-50 in tetrahydrofuran (1.0 mL) was added to the chitosan solution and continued stirring at room temperature

(30±1 °C) for 24 h. Diclofenac (20 mg) was separately dissolved in 5 mL methanol and mixed with 1.0 mL PEG-200 and was then added to the chitosan solution and stirred at room temperature (30±1 °C) for another 24 h. The resulting mixtures were casted on a petri dish and spread manually to the outside borders. The films were dried in an oven at 40 °C for 48 h.

2.6.3.3 Chitosan films cross-linked with EGDE containing

Chitosan films cross-linked with EGDE were prepared by dissolving chitosan (1.50 g) in 1% acetic acid solution (100 mL) at room temperature (30±1 °C) with magnetic stirrer for 24 h. Solution of EGDE (0.54 mL, 0.0035 mol) was added to the chitosan solution and continued stirring at room temperature (30±1 °C) for 24 h. Diclofenac (20 mg) was separately dissolved in 5 mL methanol and mixed with 1.0 mL PEG-200 and was then added to the chitosan solution and stirred at room temperature (30±1 °C) for another 24 h. The resulting mixtures were casted on a petri dish and spread manually to the outside borders. The films were dried in an oven at 40 °C for 48 h.

2.6.4 Physicochemical characterization of the chitosan films containing diclofenac

2.6.4.1 Morphology observations

The surface morphologies of chitosan films uncross-link, chitosan films cross-linked with dP-ENR-25, dP-ENR-50 and EGDE containing diclofenac were examined using scanning electron microscopy (SEM, Quanta400, FEI, Czech Republic). Prior to observation, samples were mounted on metal grids, using double-sided adhesive tape, and coated by gold under vacuum before observation.

2.6.4.2 Fourier-transform infrared (FTIR) spectroscopy

The infrared spectra of the films were obtained in a range of 4000-400 cm^{-1}, using the spectrum one FT-IR spectrometer (Perkin Elmer, USA) with ATR accessory with ZnSe crystal.

2.6.4.3 X-ray diffraction (XRD)

The physical state of the chitosan films containing diclofenac were assessed by XRD studies. X-ray diffraction spectra of chitosan films were obtained at room temperature by using a X-ray diffractometer (X′pert MPD, Philips, Netherlands), with Co as an anode material and graphite monochromator, operated at a voltage of 40 kV. The sample were analyzed in the 2θ angle in a range of 5-90° and the process parameters were set as a step size of 0.05° (θ) and scan step time of 1.0 sec.

2.6.4.4 Differential scanning calorimetry (DSC)

The DSC thermograms of pure diclofenac and chitosan film uncross-linked, chitosan film cross-linked with dP-ENR-25 or dP-ENR-50 containing diclofenac, chitosan film cross-link with EGDE containing diclofenac were obtained using DSC7, Perkin Elmer, USA. The sample of 2–4 mg was accurately weighed into an aluminum pan with cover sealed. The measurements were performed under nitrogen purge over 20–500 °C at a heating rate of 10 °C/min.

2.6.5 Determination of drug loading efficiency in the chitosan films

Contents of diclofenac entrapped in the chitosan films were determined by solvent extraction process by using methanol as a solvent. The concentrations of extracted diclofenac were analyzed by UV-Vis spectrophotometer. The analytical method was validated and the validation parameter are summarized in an appendix. The determination procedure is as following. Chitosan films cross-linked with dP-ENRs or EGDE

or uncross-linked chitosan films with drug (100 mg) were placed in 50 mL methanol in a 100 mL erlenmeyer flask and sonicated for 60 min and left standing at room temperature (30 ± 1 °C) for 60 min. The clear supernatant was taken to determine the amount of diclofenac by using UV spectrophotometer (Hewlett Packard 8452A, Diode Array Spectrophotometer, USA) ($\boldsymbol{\lambda}$ = 278 nm). Diclofenac content was calculated by comparison with the standard solutions of diclofenac in methanol. The drug loading efficiency (%) were calculated using the following equations according to the report of Wang et al., 2009. Three determinations were carried out for each formulations (n = 3).

$$\text{Drug loading efficiency (\%)} = \frac{\text{Actual diclofenac content}}{\text{Amount of diclofenac added in each formulation}} \text{X } 100$$

2.6.6 *In vitro* release study

The release study was performance using modified Franz diffusion cells (Hanson Research Corporation, USA). The diffusion area of the cells was 1.77 cm^3 and the receptor compartment volume was 12 mL. The diffusion cells were connected with a circulating water bath and the temperature was controlled at 37 °C. Phosphate buffer (PBS) pH 5.5 was used as a receptor fluid and stirred by externally driven Teflon-coated magnetic bar at 150 rpm. The diclofenac loaded films was placed on the receptor compartment, then the donor compartment was connected with a clamp. At suitable time intervals at 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, and 360 min, 1.0 mL of solution from the receptor compartment was taken, and replaced with the same volume of PBS at 37 °C. Concentration of model drug in PBS of the receptor compartment was determined by HPLC (Agilent 1100, diode array detector). The HPLC chromatographic system used for determined including:

Column: a reversed phase ACE C8, 5 µm, 4.6 x 150 mm HPLC column

Mobile phase composition: A mixture of methanol: phosphate buffer pH = 2.5 (66: 34 by volume)

Flow rate: 1.0 mL/min.

Detector: Detection of diclofenac was performed by measuring the absorption at 254 nm.

Injection volume: 20 µg/mL.

The HPLC method was validated according to the ICH guideline (I. C. H. Harmonized Tripartite Guideline, 1997), and the validated parameter are summarized in an appendix.

In order to understand the kinetic and mechanism of drug release. Six determinations were carried out for each formulations (n = 6). In the model-dependent approach, various kinetic models were applied to the release profiles, as in Eqs 1 – 5.

First order: $\ln M_t = \ln M_o + K_1 t$ ..... (1)

Higuchi: $M_t = M_o + K_H t^{1/2}$ ..... (2)

Hixson-Crowell: $M_o^3 - M^{t3} = K_{HC} t$ ..... (3)

Baker-Lonsdale: $\frac{3}{2}[1 - (1 - \frac{\mathbf{M_t}}{\mathbf{M_\alpha}})^{2/3}] \frac{\mathbf{M_t}}{\mathbf{M_\alpha}} = k_t$ ..... (4)

Korsmeyer-Peppas: $\frac{\mathbf{M_t}}{\mathbf{M_\alpha}} = K_k t^n$ ..... (5)

where, M_t is the cumulative amount of drug released at any specified time point and M_o is the initial amount of drug in the formulation. K_1, K_H, K_{HC} and K_k are rate constants for first order, Higuchi, HixsonCrowell and Korsmeyer-Peppas models, respectively. In Eq 5, $\frac{\mathbf{M_t}}{\mathbf{M_\alpha}}$ is the percentage of diclofenac released at time t and n is the release exponent that characterizes different release mechanisms (Dash *et al.*, 2010).

2.6.7 Determination of drug distribution uniformity.

The uniformity of drug distribution was evaluated using method modified from Amnuaikit *et al* (2005) by determining drug content at different places of the films by a spectrophotometric method. A known weight of film which was cut from different part of films and placed in 50 mL of methanol in a 100 mL erlenmeyer flask and sonicated for 60 min and left standing at room temperature (30$\pm$1 ^{o}C) for 60 min. The clear supernatant was taken to determine the amount of model drug by using UV spectrophotometer ($\boldsymbol{\lambda}$ = 278 nm). The drug distributions were calculated using the following equations by comparison the absorbance value of each sample with the absorbance values of standard solutions (Wang *et al.*, 2009). Nine determinations were carried out for each formulations (n = 3).

$$\text{Drug content in each sample} = \frac{\text{Actual drug content found in each sample}}{\text{Theoretical drug content in each sample calculated by using wt of the film sample}} \text{X } 100$$

2.6.8 Determination of diclofenac stability in chitosan films formulation.

The stability of the drug remaining during storage in the films were performed using the method described by cervera *et al* (2004). The film samples were stored in desiccators at 40$\pm$0.5 ^{o}C/75% RH, 50$\pm$0.5 ^{o}C/75% RH and 70$\pm$0.5 ^{o}C/75% RH (RH = relative humidity) for 3 months (3 samples). The remaining amounts of the diclofenac in each sample after storage at 1, 2 and 3 months were determined by using HPLC (Agilent 1100, diode array detector) method.

2.7 Result and discussion

2.7.1 Optimization for suitable quantity of diclofenac for chitosan film preparation.

The optimum quantity of diclofenac in films preparation were varied at 1.0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.0, 20.0, 22.5, 25.0, 27.5, 30.0, 32.5 and 35.0 mg in mixed solutions (5.0 mL methanol mixed with 1.0 mL PEG-200). A suitable formulation was selected form the formulation that did not give any precipitation in the resulting films. The results (Table 16) demonstrated that when using diclofenac more than 20.0 mg resulting in crystalline formation on the films surface. Therefore, 20.0 mg of diclofenac was chosen.

2.7.2 Optimization for a suitable volume of plasticizer (PEG-200) for chitosan film preparation.

PEG-200 was used in film formulation as plasticizer and co-solvent for diclofenac. Therefore, suitable volume of PEG-200 was investigated. PEG-200 was added to the solution of diclofenac in methanol in varying volume from 0.25-2.00 mL before adding to the chitosan with dP-ENR solution. A suitable volume was selected from the maximum volume that gave transparent film with good flexibility and no drug precipitate was observed. The results (Table 17) showed that when PEG-200 was added to the formulation from 0.25-0.75 in both formulations, diclofenac was found as precipitate in the dried films. That could be due to insufficient volume of PEG-200 to promote diclofenac solubility during film drying process. Yellow transparent films without precipitate were obtained when using PEG-200 from 1.00 mL and over, however, when PEG-200 was added from 1.25-2.00 mL, the excess of PEG-200 was observed to be remained on the films as droplets like. Therefore, 1 mL of PEG-200 was selected in the chitosan film formulation preparation. The resulting films were yellow transparent without precipitation with good flexibility.

Table 16 Optimization for suitable amount of diclofenac for chitosan film formulation.

Diclofenac (mg)	Observation of chitosan films cross-linked with dP-ENR-25	Observation of chitosan films cross-linked with dP-ENR-50
1.0	yellow transparent film	yellow transparent film
2.5	yellow transparent film	yellow transparent film
5.0	yellow transparent film	yellow transparent film
7.5	yellow transparent film	yellow transparent film
10.0	yellow transparent film	yellow transparent film
12.5	yellow transparent film	yellow transparent film
15.0	yellow transparent film	yellow transparent film
17.5	yellow transparent film	yellow transparent film
20.0	yellow transparent film	yellow transparent film
22.5	Drug powder distributed on film	Drug powder distributed on film
25.0	Drug powder distributed on film	Drug powder distributed on film
27.5	Drug powder distributed on film	Drug powder distributed on film
30.0	Drug powder distributed on film	Drug powder distributed on film
32.5	Drug powder distributed on film	Drug powder distributed on film
35.0	Drug powder distributed on film	Drug powder distributed on film

2.7.3 The suitable formulations for chitosan film cross-linked with dP-ENRs containing diclofenac.

From all investigations, the suitable formulations for chitosan films cross-linked with dP-ENRs are summarized in Table 18. The procedures for chitosan film preparation were using 1.5 g of chitosan (MW 300K) dissolved in 100 mL of 1.0% of acetic acid and mixed with 1.0% dP-ENR-50 (1 mL) or 4% dP-ENR-25 (1 mL) and stirred at RT for 24 h. Diclofenac (20 mg) was separately dissolved in 5 mL methanol and mixed with PEG-200

1 mL until clear solution was obtained. The latter solution was added to the former solution while stirring and continued stirring at RT for another 24 h. The resulting solutions were then casted on petri dish (Ø 9 cm) and dried in oven at 40 °C for 48 h.

Table 17 Observation result of varying volume of PEG-200in chitosan film formulation.

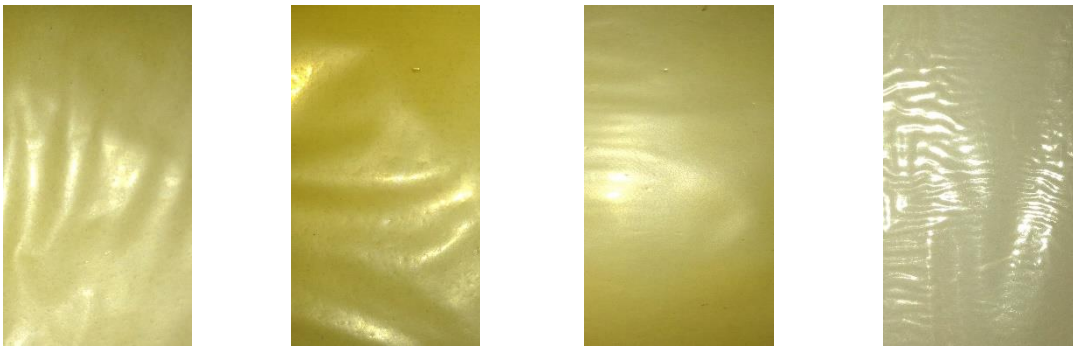
Volume of PEG-200 (mL)	Observation of chitosan cross-linked with dP-ENR-25	Observation of chitosan cross-linked with dP-ENR-50
0.25	Yellow transparent film with precipitation	Yellow transparent film with precipitation
0.50	Yellow transparent film with precipitation	Yellow transparent film with precipitation
0.75	Yellow transparent film with precipitation	Yellow transparent film with precipitation
1.00	Yellow transparent film without precipitation	Yellow transparent film without precipitation
1.25	Yellow transparent film without precipitation	Yellow transparent film without precipitation
1.50	Yellow transparent film without precipitation	Yellow transparent film without precipitation
2.00	Yellow transparent film without precipitation	Yellow transparent film without precipitation

Table 18 Suitable compositions for chitosan films cross-linked with dP-ENRs containing diclofenac

Materials	
Chitosan (MW 300K)	100 mL of 1.50% in 1.0% acetic acid
4.0% dP-ENR-25 in THF	1.0 mL
1.0% dP-ENR-50 in THF	1.0 mL
Diclofenac	20.0 mg
Methanol	5 mL
PEG-200	1 mL

2.7.4 Characteristic observation of chitosan films

Different formulations were used to prepare chitosan films and the obtained films were observed for their characteristics. Figure 27 shows the characteristics of 4 different films obtained from 4 different formulations. All films displayed as yellow transparent films with some wrinkles.



(a) (b) (b) (d)

Figure 27. Pictures of the obtained films after casting: (a) uncross-linked chitosan film with diclofenac (CH-film); (b) CH-dP-ENR-25 with diclofenac; (c) CH-dP-ENR-50 with diclofenac; (d) CH-EGDE with diclofenac.

2.7.4.1 Morphology observations

Scanning electron microscope (SEM) was used to study the microstructural morphology of uncross-linked chitosan film, chitosan films cross-linked with dP-ENRs or EGDE loaded with diclofenac. Figure 28a-b were SEM micrographs of surface and cross-section of uncross-linked chitosan film containing 20 mg diclofenac. Diclofenac crystal was observed in needle form distributed on the film's surface. The crystals were found near the surface of the films which could be due to the crystal of diclofenac was formed faster near the surface during solvent evaporation. None of any crystal was observed in all cross-linked chitosan films (Figure 28c-h). Chitosan film cross-linked with dP-ENR-25 showed spherical particle about 2 μm disperse along the film surface. In chitosan film cross-link with dP-ENR-50, no particle was observed but the surface of the film displayed irregular compact structure. Chitosan film cross-linked with EGDE showed small nodules distributed along the film surface. The cross-section of chitosan films cross-linked with dP-ENR-25 and dP-ENR-50 and chitosan film cross-linked with EGDE showed a smooth appearance and good compact structure without any precipitation or aggregation.

2.7.4.2 Fourier-transform infrared (FTIR) spectroscopy of the obtained films.

The compatibility of the drug with the film compositions was studied by FTIR analysis. FTIR spectrum of pure diclofenac (Figure 29a) exhibited distinctive peaks at 3323.5 cm^{-1} due to N-H stretching of secondary amine, at 1694.2 cm^{-1} owing to –C═O stretching of carboxyl ion, and 740.7 cm^{-1} due to C–Cl stretching. Generally, 3 to 4 peaks in the range of 1400 cm^{-1} - 1550 cm^{-1} indicate the presence of aromatic ring. The spectrum of diclofenac showed peaks at 1401.72 cm^{-1}, 1454.6 cm^{-1} and 1507.90 cm^{-1} confirming the presence of aromatic rings (Pal *et al.*, 2011). FTIR spectrum of diclofenac loaded matrix films both in uncross-linked and cross-linked chitosan films are displayed in Figure 29b-e. All formulations of chitosan films found that peaks of carbonyl at 1694 cm^{-1} and NH

stretching at 3323 cm^{-1} in FT-IR spectra disappeared and may be shifted to the lower frequency, which could be due to hydrogen bonding interaction between carbonyl group and NH group with the compositions in the film formulations.

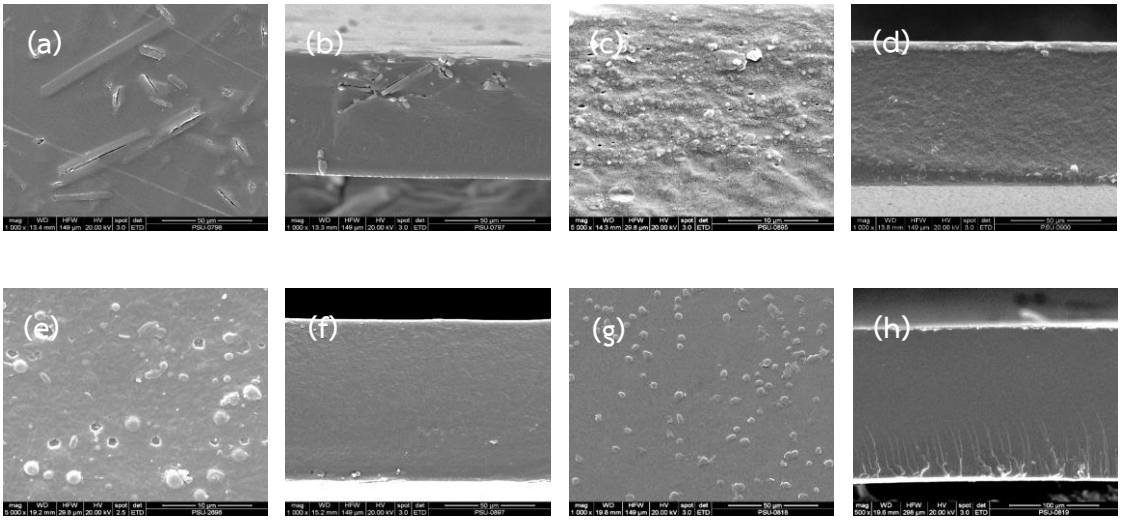


Figure 28. SEM micrographs of chitosan formulations, (a) CH-film with drug; (b) cross-section of CH-film with drug; (c) CH-dP-ENR-50-film with drug; (d) cross-section of CH-dP-ENR-50-film with drug; (e) CH-dP-ENR-25-film with drug; (f) cross-section of CH-dP-ENR-25-film with drug; (g) CH-EGDE-film with drug; (h) cross-section of CH-EGDE-film with drug.

2.7.4.3 X-ray diffraction

X-Ray diffractometry was used to determine the crystallinity properties of pure diclofenac and diclofenac in the films formulations and the results are displayed in Figure 30. The powder X-Ray diffractometry pattern of pure diclofenac shows a number of distinctive sharp peaks indicating crystalline structure (Fig. 30a). These sharp peaks of diclofenac were still observed in the XRD pattern of uncross-linked chitosan films (Fig. 30f). Therefore, diclofenac presence in chitosan uncross-linked films remained in crystalline state. The result is in accordance with the result form SEM of diclofenac in

uncross-linked chitosan film which was presented in needle form. The XRD cross-linked chitosan films with dP-ENR-25 and dP-ENR-50 (Fig. 30c, 30e), illustrated that all sharp peaks of crystalline diclofenac disappeared indicated that diclofenac changed its solid state from crystalline to amorphous form.

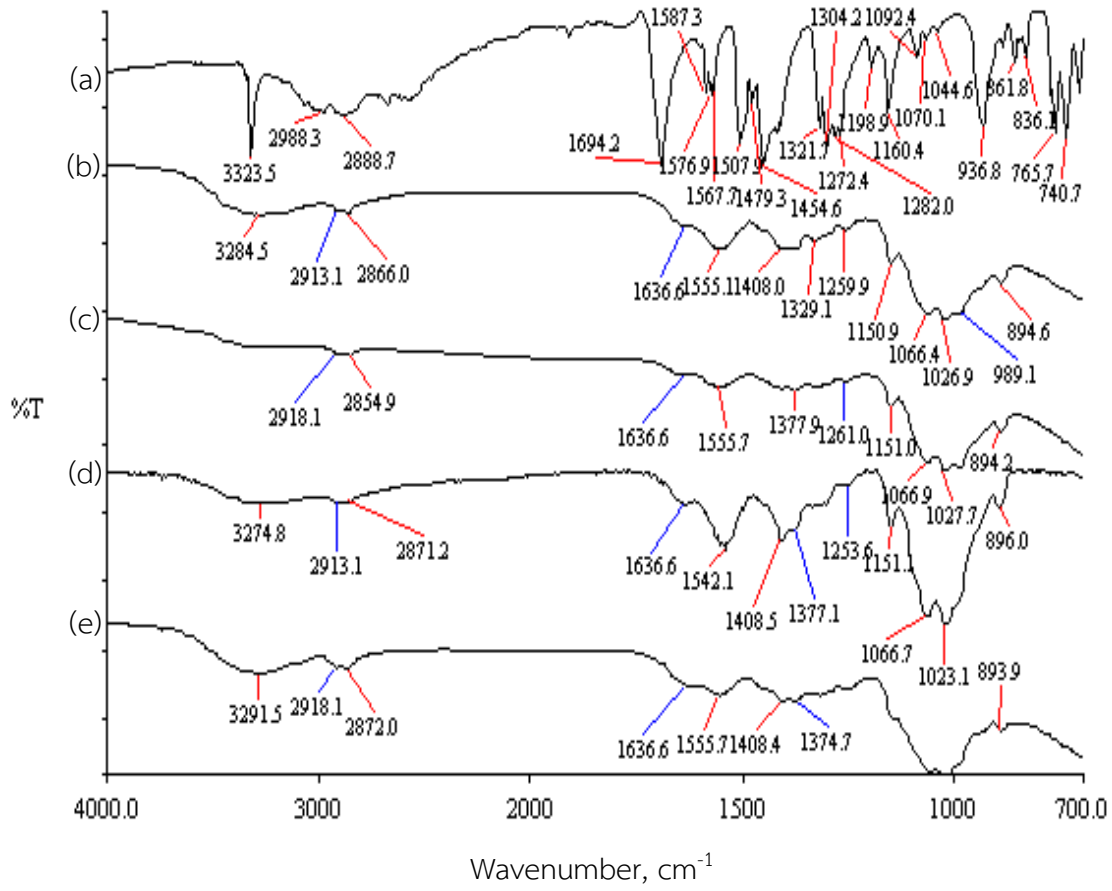


Figure 29. FT-IR spectra of (a) diclofenac; (b) CH; (c) CH-dP-ENR-25; (d) CH-dP-ENR-50; (e) CH-EGDE, all formulations with drug.

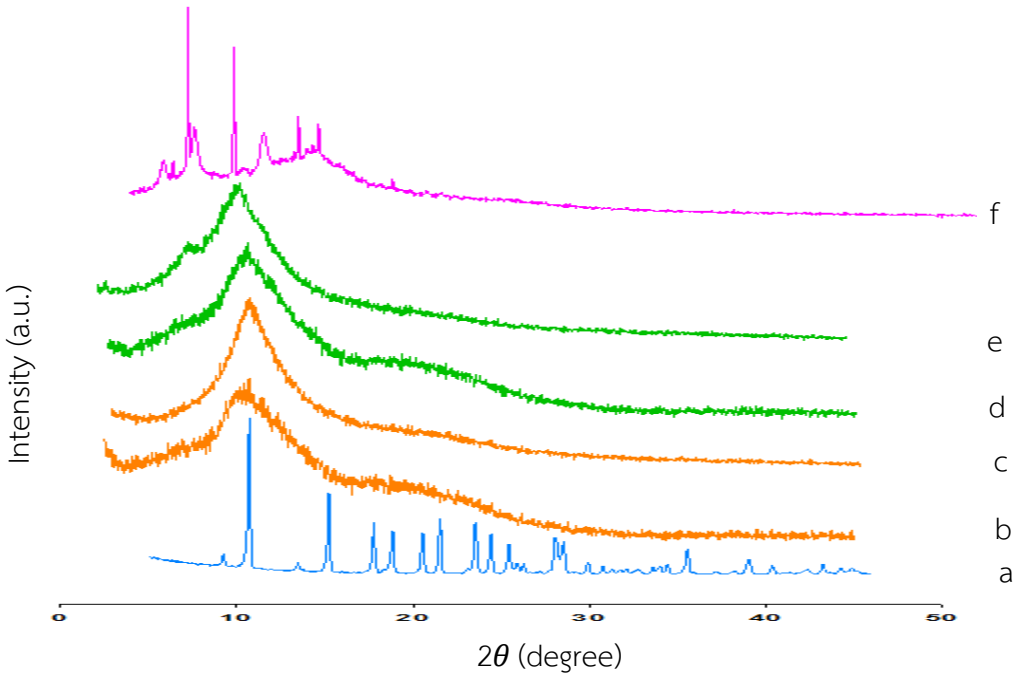


Figure 30. X-ray diffraction patterns of (a) diclofenac; (b) CH-dP-ENR-25 without drug; (c) CH-dP-ENR-25 with drug; (d) CH-dP-ENR-50 without drug; (e) CH-dP-ENR-50 with drug; (f) CH with drug.

2.7.4.4 Differential scanning calorimetry (DSC)

Differential scanning calorimetry is a thermal analysis technique used to measure changes in heat flows associated with material transition. DSC measurements could provide both qualitative and quantitative information on endothermic (heat absorption) and exothermic (heat evolving) process. DSC is commonly utilized to determine the glass transition temperature and crystalline melting point of polymeric material. In this study all samples including pure diclofenac and films were subjected for DSC analysis. DSC thermogram of pure diclofenac shows a melting endotherm at about 172.6 °C (Figures 31). The DSC thermograms of films showed two peaks, the first broad endothermic peak at 100 °C was due to water loss (Iturriaga *et al.*, 2014, Pereira Jr *et al.*,

2015) and the second broad exothermic peak at 283 °C due to the degradation of chitosan suggested by Shivashankar and Mandal, 2013. In the case of drug-loaded films DSC thermograms showed no peak corresponding to diclofenac, indicating the amorphous dispersion of diclofenac in the polymer matrix.

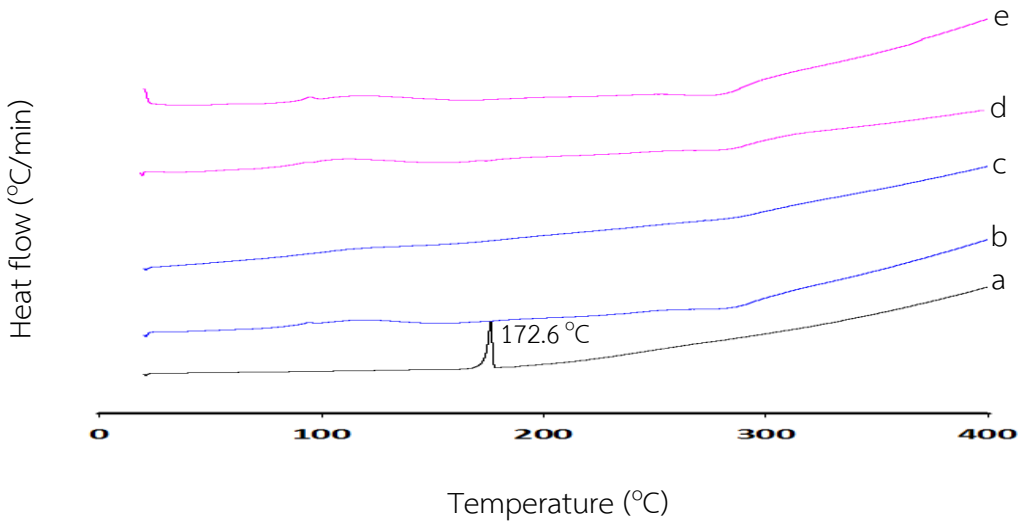


Figure 31. DSC thermogram of (a) diclofenac, (b) CH-dP-ENR-25 without drug, (c) drug-loaded CH-dP-ENR-25, (d) CH-dP-ENR-50 without drug and (e) drug-loaded CH-dP-ENR-50.

2.7.5 Determination of drug entrapment efficiency in film formulation

The drug presents in the chitosan film, chitosan films cross-linking with dP-ENR-25 and dP-ENR-50 containing diclofenac must be completely extracted by suitable method during analytical process. The entrapped drug was extracted by using methanol and determined by UV-Vis spectroscopy (λ = 278 nm) as described in an appendix. The drug entrapment efficiency in films are summarized in Table 14. High drug entrapment

efficiencies were observed in all film formulations. The amounts of drug loaded were in the range of 95.00±0.56% and 96.00±1.62 %.

Table 19 %drug entrapment efficiency of diclofenac in films

Formulations	% drug entrapment in chitosan films (n = 3, mean±SD)
CH	95.00±1.26
CH-dP-ENR-25	95.66±1.01
CH-dP-ENR-50	96.00±1.62
CH-EGDE	95.35±1.80

2.7.6 *In vitro* release studies (n = 6)

Drug release profile was studied by using franze diffusion apparatus by using film placing between upper and receiver site. Phosphate buffer (0.2 M, pH 5.5) was placed in receiver site and control the temperature constant at 37 °C. The solution from receiver site was sampling (1 mL) at selected time interval for 6 h. Every time the solution was taken new PBS was replace 1 mL. The sampling solution was determined for the diclofenac content by using HPLC (Agilent 1100 series, Germany). The cumulative dissolution profiles of diclofenac uncross-linked chitosan film (CH-film), chitosan film cross-linked with EGDE (CH-EGDE), chitosan film cross-linked with dP-ENR-25 (CH-dP-ENR-25) and chitosan film cross-linked with dP-ENR-50 (CH-dP-ENR-50) are displayed in Figure 32. Diclofenac release profile from uncross-linked chitosan film showed fast release. Diclofenac was found to reach 100% within 45 min. Slower release rate was observed from the chitosan cross-linked with EGDE film. The maximum release was detected at 2 h. However, sustained release profiles were observed from both formulations of chitosan film cross-linked with dP-ENRs. All entrapped drug was completely released at 6 h. Slightly

lower release rate was observed in the chitosan film cross-linked with dP-ENR-50 than dP-ENR-25. However, the release profiles from both formulations were similar.

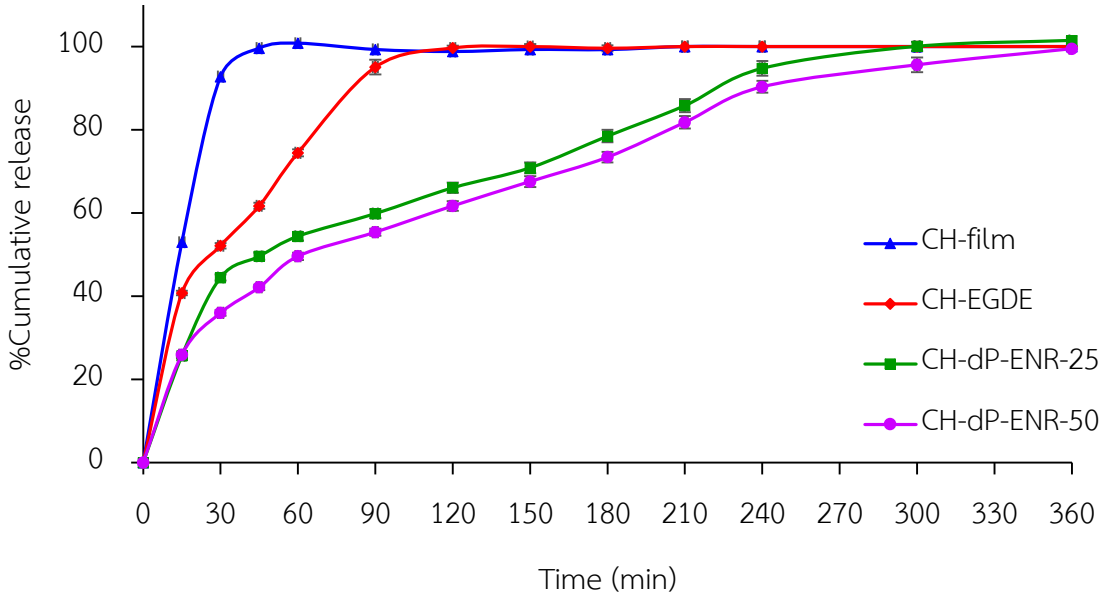


Figure 32. Dissolution profiles of diclofenac from different formulations of chitosan films (n = 6).

2.7.7 Release kinetics

The dissolution data of all formulations were treated with five different mathematical models, namely, First order kinetic model, Hixon and Crowell model, Higuchi model, Baker and Lonsdale model and Korsemeyer-peppas model. The best fit model will give the highest regression coefficient. The result demonstrated that the release kinetic of diclofenac from uncross-linked chitosan film and chitosan cross-linked with EGDE were first order kinetic. Therefore, the drug release was depend on the drug

concentration in the films. However, the release kinetic of diclofenac from chitosan cross-linked with both dP-ENRs were fitted with Korsemeyer-peppas model in Fickian diffusion pattern indicated that the drug was diffused through the porous network of the film (Korsmeyer *et al.*, 1983).

In uncross-linked chitosan film, most of the entrapped drugs did not have strong interaction with chitosan in film formulation or the entrapped drug were adsorbed on the surface of the film. That may give rise to the rapid release of the drug from the film. Slightly stronger interaction between diclofenac with film compositions in CH-EGDE film, hence, slower release was observed. In contrast to the release pattern of diclofenac from CH-dP-ENRs films, diclofenac was strongly interacted with the film compositions. Moreover, the drug could be also entrapped in the 3D-network formed by cross-linked reaction, therefore, much slower released of diclofenac from CH-dP-ENRs films were noticed.

2.7.8 Drug distribution in film

Estimation of drug content at different places on each film indicated that diclofenac was distributed uniformly throughout the films. The drug distribution efficiency in the films are summarized in Table 21. The amounts of drug loaded at different placed of the films were in the range of 91.85 and 94.85%. The results indicated that the drug was well dispersed throughout the films during casting and drying process.

Table 20 Kinetics analysis of the release data of diclofenac from chitosan films treated with kinetic equation models*

Formulations	First order		Hixon and Crowell		Higuchi model		Baker and Lonsdale		Korsemeyer-peppas model		
	r^2	K	r^2	k	r^2	k	r^2	k	r^2	K	n
CH-dP-ENR-25	0.9345	0.0113	0.8895	0.0029	0.9611	5.9323	0.9835	0.0011	0.9899	10.7885	0.3861
CH-dP-ENR-50	0.9522	0.0094	0.9197	0.0025	0.9861	5.6143	0.9865	0.0009	0.9959	8.2544	0.4267
CH-EGDE	0.9762	0.0256	0.9712	0.0068	0.9172	8.6782	0.9733	0.0027	0.9648	16.1086	0.3671
CH-uncross-linked	0.9575	0.0628	0.8768	0.0063	-1.6167	9.7541	0.3986	0.0036	0.7312	49.3830	0.1488

*Sigma plot v.12 was used for the data treatment

Table 21 %drug entrapment efficiency of diclofenac in films at different parts of the films

Formulations	%drug entrapment efficiency of diclofenac in films		%RSD
CH-film uncross-linked	Sample 1	92.76	0.53
	Sample 2	91.85	
	Sample 3	92.61	
CH-EGDE-film	Sample 1	93.55	0.32
	Sample 2	93.96	
	Sample 3	93.38	
CH-dP-ENR-25-film	Sample 1	94.85	0.34
	Sample 2	94.23	
	Sample 3	94.78	
CH-dP-ENR-50-film	Sample 1	94.28	0.45
	Sample 2	94.01	
	Sample 3	94.83	

2.7.9 Stability study of diclofenac in film formulation during storage

The stability of diclofenac in films formulation were determined by storage the films samples in climatic stability chamber at 40 ºC, 50 ºC and 70 ºC in 75% relative humidity for 90 days. At selected time intervals, the film samples were taken from the stability chamber and determined for the amount of diclofenac remaining in the films by UV-vis spectrophotometer and the results are displayed in Figures 33-35. The result demonstrated that diclofenac in all film formulations were degraded during storage time for 90 days. Storage condition at higher temperature (70 ºC) will promote the degradation of diclofenac than that at lower temperature (40 ºC and 50 ºC). At 40 ºC, diclofenac in all film formulations was degraded slowly in the same pattern. Diclofenac in uncross-linked chitosan film and CH-dP-ENR-25 degrade slowly and at 90 days of the storage time, the mount of diclofenac were still remained above 90%. However, diclofenac in CH-dP-ENR-50 was found to degrade faster during 60-90 days of the storage time and gave only 88.18% remaining. At 50 ºC, diclofenac was faster degraded in all film formulations. Diclofenac in uncross-linked chitosan film was degrade faster than that in CH-dP-ENRs films. The degradation profiles of diclofenac in CH-dP-ENRs films were similar. However, diclofenac contents were less than 80% after storage for 90 days. At 70 ºC, very high degradation of diclofenac was observed in all film formulations. Both CH-dP-ENRs films gave the same pattern in drug degradation. Faster degradation of diclofenac was detected in the uncross-linked CH-film. At 70 ºC, the drug could preserve their stability less than 10 days. From these results indicated that the films should be kept at low temperature in dry place in order of preserve the stability of the drug in the formulations.

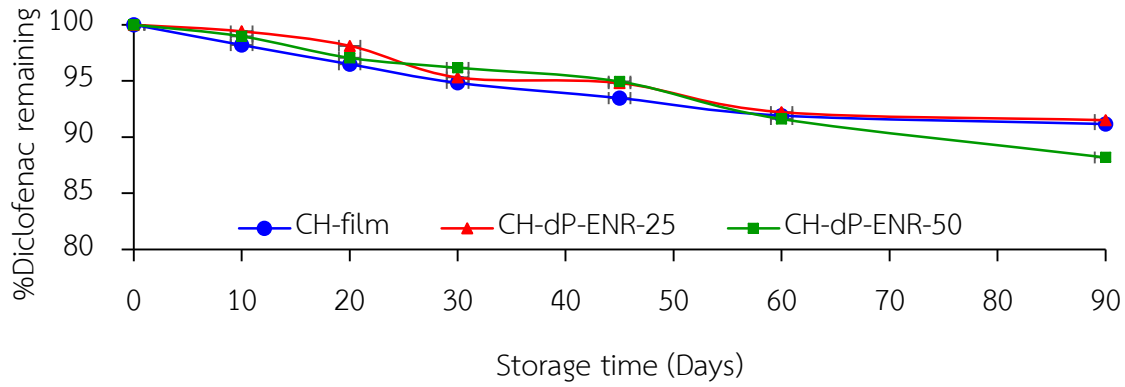


Figure 33. Stability of diclofenac in chitosan films formulations at 40 ºC (75% RH) during 90 days of storage time.

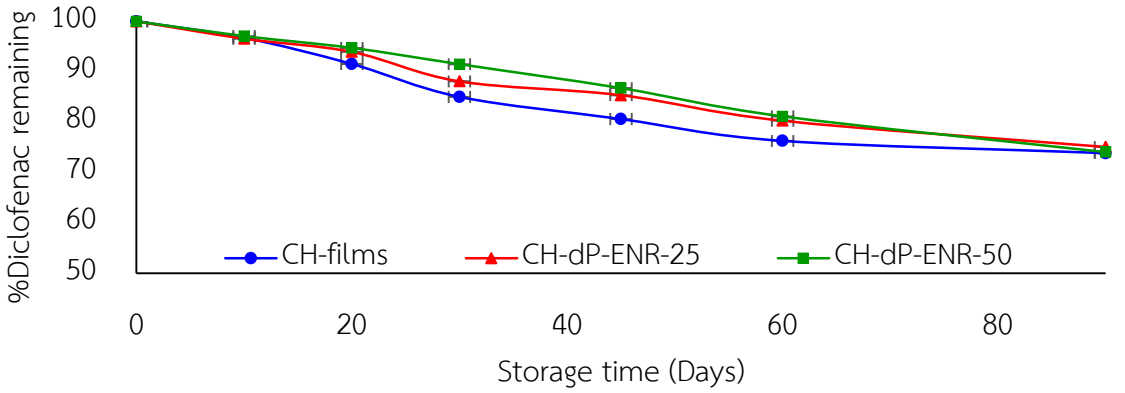


Figure 34. Stability of diclofenac in chitosan films formulations at 50 ºC (75% RH) during 90 days of storage time.

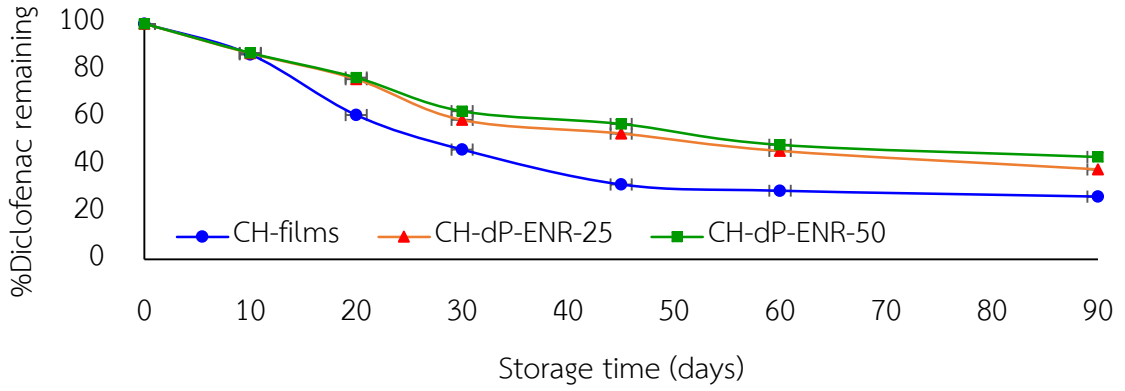


Figure 35. Stability of diclofenac in chitosan films formulations at 70 ºC (75% RH) during 90 days of storage time.

2.8 Conclusions

Chitosan films can be prepared successfully by cross-linking reaction using dP-ENR as cross-linker. Both dP-ENR-25 and dP-ENR-50 can be used in chitosan film preparation as cross-linkers. The resulting products were found to have better mechanical property for example, better swelling property, higher moister uptake capacity, increate TS and elongation at break. They also demonstrated to have no skin irritation. The cross-linking reaction should occur via nucleophilic ring opening reaction of epoxide ring of ENR provide three dimension network with improved elasticity and hydrophilicity of chitosan film.

The films of chitosan cross-linked with dP-ENR containing diclofenac drug were successfully obtained by casting method. High drug entrapment efficiencies were observed in all film formulations and the drug was well distributed throughout the films. FT-IR results demonstrated that the drug molecule has interaction with chitosan's moieties, especially by hydrogen bonding interaction. The drug was well dispersed within the films in amorphous form indicated by DSC and XRD results. CH-dP-ENRs films provided sustained release patterns of the drug from the film whereas uncross-linked CH-film and CH-EGDE film gave fast drug release pattern. Diclofenac was released from CH-dP-ENRs films in Korsemeyer-peppas model which the interaction of the drug and film compositions played an important role in drug solubility property. The dissolved drug diffused from the porous network of the film during solubility study. To preserve drug stability in the chitosan films, the films should be stored in low temperature (< 40 ºC) in a controlled humidity.

CHAPTER 3

CHITOSAN MICROSPHERES

3.1 INTRODUCTION

One of the novel drug carrier dosage forms is microsphere. It has become the drug delivery advantageous recently. Microspheres can be identified as micro particular dosage. It is the form for delivering system for many active ingredients. They could manage the delivery to the site of action in body (Turkoglu *et al.*, 2005). The particle size are normally distributed in between 0.02 to 1000 μm. They are the systems' of spherical and solid drug delivery which may contain micro porous in the structure (Sudhamani *et al*., 2010, Saralidze *et al*., 2010). Since 1980s, Chitosan microspheres have been developed extensively (Williams *et al.*, 1998). It is observed that the delivery of drug of chitosan microspheres is suitable for both local and systemic therapies (Sudhamani *et al.*, 2010). Chitosan microspheres can provide a controlled release pattern. They can be applied for enzymes, proteins, vitamins, and hormones delivery (Zhou *et al.*, 2006).

This system demonstrated to promote bioavailability of drugs. It also increases the uptake of several hydrophilic substances. They are done through the epithelial layers with enhanced prenetration. It is stable against the stomach harsh environment upon the oral administration (Jose *et al.*, 2013). Chitosan is also believed to improve the absorption of the drug through tight junctions via the route of paracellular. It will be done by the direct interaction of cationic polymer molecules with the negatively charged cell membrane (Wang *et al.*, 2006). Chitosan microspheres containing magnetic microspheres (Denkbaş *et al*., 2002, Dudhani and Kosaraju, 2010) and

diltiazem hydrochloride (Jose *et al.*, 2011) have been prepared and described for site-specific delivery.

Moreover chitosan microspheres can be designed to give the sustained release against a prolonged time period. It could improve bioavailability of many active compounds than the conventional dosage forms, therefore the dosing frequency can be reduced. It also can decrease side effects and increases compliance of the patient. The large surface area of microspheres could enhance bioavailability, since microspheres are normally in spherical shape and have the smaller size. They have an advantage over the microparticles in site specific drug delivery since they tend to accumulate at the action size as its size is smaller. Moreover, they can be injected due to their spherical shape (Prasanth *et al.*, 2011). Various available reports are on chitosan microspheres' preparation and development for drug delivery application. The development of process to prepare the devices of microparticulate polymeric drug delivery can be utilized for chitosan microsphere preparation. There are four important approaches, such as an ionotropic gelation with oppositely charged, the complex and simple coacervation, solvent or emulsification evaporation and spray drying method (Oliveira *et al.*, 2005).

3.1.1 Preparation methods of chitosan microspheres

Various methods in chitosan microspheres preparation have been reported. Selection of proper preparation methods for the desired chitosan microspheres depends on several factors. For example, the requirement of particle size, an active agents' chemical and thermal stability, the release kinetic profile reproducibility, and final product stability. The toxicity of the final product should also be considered. The reported methods which have been used in the chitosan microsphere preparation are discussed below.

3.1.1.1 Cross-linking emulsion

Water-in-oil (w/o) emulsification method is the method of microsphere construction by emulsifying the solution of aqueous chitosan in oil phase. Stabilized aqueous droplets are done by suitable surfactant. By using a suitable cross-linking agent, stable emulsion will be cross-linked. After stirring, the droplets of chitosan microspheres were obtained by hardening process.

Microspheres are repeatedly filtered and washed. The washing solvent was the proper organic solvent such as n-hexane, following by alcohol and lastly dried. The method is schematically represented in Figure 36 (Agnihotri *et al.*, 2004). For application of chitosan microspheres the delivery of protein by using genipin as a cross-linking agent, can be utilized. The obtained chitosan microspheres are normally spherical in shape. The size of the microspheres are acceptable in a range of 80 and 100 μm. Microspheres cross-link with genipin demonstrated that protein release from chitosan microspheres was in a controlled release pattern (Karnchanajindanun *et al.*, 2011). Ramakrishna *et al*., 2011 prepared acebutolol HCl loaded chitosan microspheres using the method of water-in-oil (w/o) emulsion. They are cross-linked with the glutaraldehyde. Acebutolol HCl can be released from the microspheres of chitosan in a pattern of the controlled release. Chitosan microspheres containing haparin were constructed by the technique of water-in-oil emulsification solvent evaporation procedure. Diameters of the resulting microspheres are controllable. They have smooth and spherical surface morphology (He *et al.*, 2007). These microspheres were found to be able to be used for active drug delivery system.

Emulsification of cross-linked chitosan microspheres with glutaraldehyde could produce chitosan microspheres for chronic stable angina chronotherapy. The chitosan microspheres' particle size were between 6.32 μm and 9.44 μm (Jose *et al.*, 2013). Moreover, the method for preparation chitosan microspheres by 'dry-in-oil multiple emulsion' process proposed for loading a lipophilic drug into chitosan microspheres. It was based on of o/w/o multiple emulsion formation. This method was a good technique to encapsulate hydrophobic active substances in hydrophilic polymers. The

chitosan microspheres with narrow size and good morphological characteristics were acquired without utilizing any cross-linking agent (Genta *et al.*, 1997). Zhou *et al* (2006) prepared chitosan or cellulose acetate multi microspheres by w/o/w emulsion method. Hydrophilic drugs (ranitidine hydrochloride) were loaded. The results demonstrated that the microspheres were spherical, having size in a range of 200–280 μm and the drug was controlled release from the microspheres. Peng *et al* (2006) used *N*-methylated chitosan to prepare microspheres by cross-linking reaction with glutaraldehyde. The resulting chitosan microspheres was used to encapsulate ofloxacin. The results showed that microspheres had exhibited much smooth surface. Ofloxacin encapsulated in the microspheres was released rapidly into the solution of phosphate buffer (pH 7.4). It was released slowly in 0.1 M HCl (pH 1.2). The microsphere release mechanism was observed to be non-Fickian diffusion pattern. Membrane emulsification technique combined with the method of thermal-gelation had successfully created pH sensitive chitosan microspheres (Wu *et al.*, 2008). In this study, bovine serum albumin was encapsulated as a model drug in the microspheres. It was found slowly released in the neutral medium and rapidly released in acid solution. These chitosan microspheres are pH sensitive and can be utilized as the system of drug delivery in biomedical field such as the carrier of tumor-targeted drug. Uniform-sized chitosan microspheres containing insulin as a model protein active agent were constructed by the combination of stepwise solidification method and the technique of membrane emulsification (Wang *et al*., 2006a and 2006b). The poor reproducibility disadvantages and the disadvantages of the broad size distribution which normally observed in the conventional mechanical stirring emulsification. The microspheres were used to prepare chitosan droplets which was previously prepared by step-wise solidification can overcome high burst effect disadvantages found in conventional adsorption protocol of the protein drug in microsphere and lower in the efficiency of encapsulation.

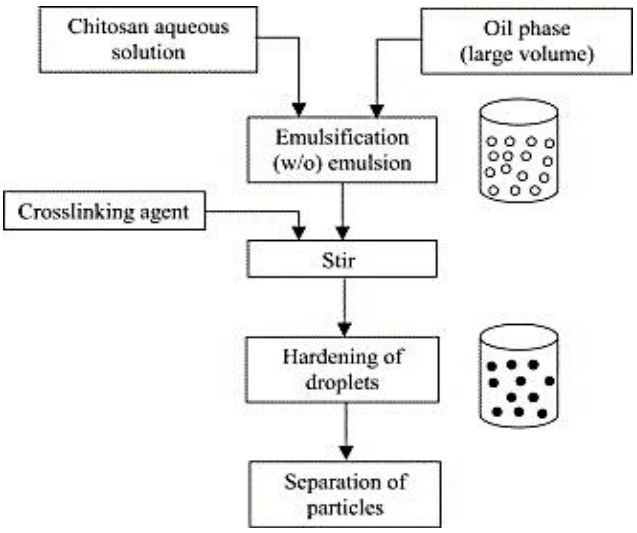


Figure 36 Schematic demonstration of chitosan microspherical systems preparation by the emulsion combined with cross-linking method (From: Agnihotri et al., 2004).

3.1.1.2 Coacervation/precipitation technique

The coacervation/precipitation method was reported as another method for chitosan microspheres preparation. In this technique, chitosan microspheres can be precipitated or coacervated when they contact with alkaline solution since chitosan is insoluble in alkaline medium. The solution chitosan was administrated into the solution of alkali to produce particles. Alkali solution, for example, ethanediamine, NaOH-methanol or sodium hydroxide solutions are usually utilized. The chitosan solution was applied to the alkali solutions by using compressed air nozzle to form coacervation droplets (Figure 37). The reported coacervation method recently has been found in the chitosan microspheres preparation. For example, microspheres of casein–chitosan loaded with diltiazem hydrochloride were arranged by dissolve diltiazem hydrochloride in acidic chitosan solution. The resulting solution was blown to NaOH solution to produce precipitation. The diltiazem hydrochloride was well incorporated into the microspheres. The dissolution profiles of diltiazem hydrochloride from casein–chitosan microspheres provided drug retarded release pattern to the distilled water medium (Bayomi *et al.*, 1998). Chitosan micro-particle encapsulation of the drug with high molecular weight, rifampicin has been

prepared using the coacervation technique. In this study, solution of sodium alginate was dripped into a chitosan solution containing $CaCl_2$ through a glass capillary and a peristaltic pump to give the microparticle of chitosan. The incorporated drug was detected inside the microparticles via the process of coacervation. The results demonstrated that chitosan/alginate micro act as an efficient delivery system for the rifampicin's controlled-release. The release profiles demonstrated that 20% drug release occurred in 2 h at acidic pH, and rapid release rate reached up to 100% at pH of 6.8 (Lacerda *et al.*, 2014).

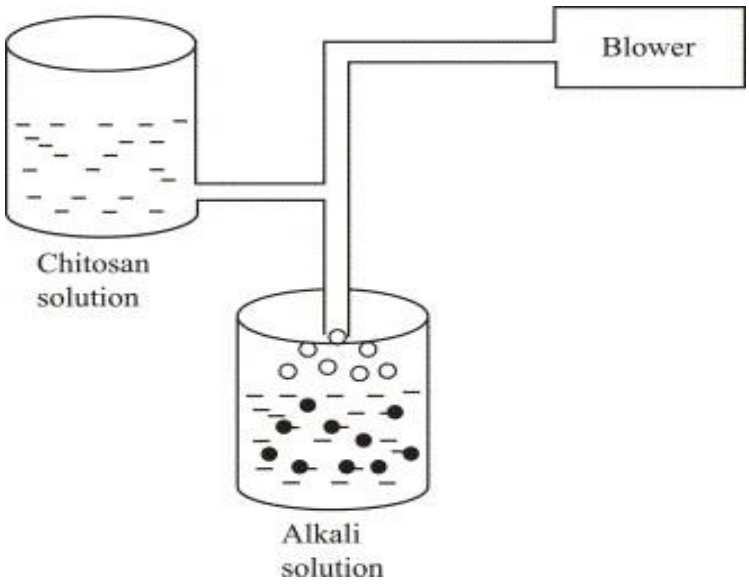


Figure 37 Picture of the systems of the chitosan microparticulate system preparation by coacervation/precipitation method (From: Agnihotri et al., 2004).

3.1.1.3 Method of Ionic gelation

The method of ionic gelation involves a complexation between the negative and positive charges of tripolyphosphates and chitosan to chitosan microspheres preparation. This technique has attracted much attention recently. Since the process is simple and can be performed under mild control conditions without

involving high temperatures, organic solvents, or sonication (Fàbregas *et al.*, 2013, de Pinho Neves *et al.*, 2014). (TPP) or Sodium tripolyphosphate is a polyanion compound. It can interact with the cationic chitosan through the forces of electrostatic (Shenvi *et al.*, 2014). The TPP-chitosan complex microspheres have been explored as pharmaceutical usage potential after Bodmeier *et al* (1989). Their preparation process was performed by dropping chitosan droplets into the solution of TPP. Chitosan is dissolved prior in the solution of aqueous acidic to get chitosan solution in the method of ionic gelation. The solution of chitosan was added dropwise under the constant stirring to the solution of TPP polyanionic. Chitosan precipitates can be formed in spherical particles and undergoes the gelation of ionic according to the complexation between positive and negative charges ingredients. The schematic preparation method is shown in Figure 38. Chitosan microspheres/TPP were found to have low mechanical strength. It also limited in drug delivery usage. Cho *et al* (2014) studied resveratrol (3,4,5-trihydroxy-*trans*-stilbene)-loaded (TPP) chitosan–sodium tripolyphosphate micro- spheres by the use of medium (190 to 310 kDa) and (310 to 375 kDa) high chitosan molecular weight. The ionic gelation method was performed using different concentrations of TPP to entrap resveratrol for bioavailability improvement. Microspheres were produced by using a 450 μm nozzle encapsulator. The mean particle size of the microspheres was between 160 - 206 μm. It exhibited the distribution of a narrow size since the increase of the concentration of TPP solution. Encapsulation efficiency was observed to be very high from 94% to 99%. Crystallinity of the microspheres was decrease when the chitosan concentration decrease from 1% to 0.5%. FTIR data showed an interaction polyelectrolyte between TPP and chitosan. The patterns of X-ray diffraction were matched with FTIR and DSC. It showed hydrogen bonding enhancement with the concentration of TPP and crystallinity decrease. An increase from 1% to 3% in the TPP solution concentration made a less initial burst of the release of the resveratrol. Curcumin-phytosome-loaded chitosan microspheres have been prepared by first dissolving curcumin in soybean phospholipids and then added to chitosan and TPP solution. The solution result was then fed through a 2 mm diameter nozzle via peristaltic pump. The curcumin-phytosome microspheres were constructed by encapsulating curcumin-phytosomes in microspheres of chitosan via

ionotropic gelation usage. The final microsphere with 23.21 ± 6.72 μm of a mean particle size having spherical in shape were obtained. Drug loading efficiency was 2.67 ± 0.23%. Fourier transform infrared spectroscopy and the differential scanning calorimetry determined that phytosomes integrity was preserved within microspheres' polymeric matrix. The *in vitro* release rate of curcumin from the curcumin-phytosome microspheres was in slow pattern (Zhang *et al.*, 2013). *Wu et al* (2008) prepared pH-sensitive uniform-sized quaternized chitosan microspheres. They were done by the combination of the method of novel thermal-gelation and the technique of Shirasu Porous Glass (SPG) membrane emulsification. Glycerophosphate and the quaternized chitosan solution mixture were prepared and dispersed in oil phase and water phase. A uniform w/o emulsion was obtained by the technique of SPG membrane emulsification. Droplets were solidified into microspheres at 37°C by the method of thermal-gelation. The process condition effect on obtained microsphere's property was observed. It also obtained the condition of an optimized preparation. The obtained microsphere showed apparent pH-sensitivity and porous structure as indicated in the result. It rapidly dissolved BSA in (pH 5) acid solution. It also kept stable in (pH 7.4) neutral solution. The result demonstrated that the pH-sensitive microspheres affected the behavior of drug release. Model drug, bovine serum albumin (BSA) was encapsulated in the microspheres. It was slowly released in neutral medium and rapidly released in the acidic medium. Gupta and Jabrail (2007) created cross-linked microspheres for the controlled delivery of hydroxy urea and rifampicin. The sodium hexametaphosphate (SHMP) anion cross linkers and (STPP) sodium tripolyphosphate were used to prepare the cross-linked microspheres. Chitosan with (1134 kg/mol) constant molecular weight and deacetylation constant degree of (75 wt %) are useful for the controlled release of the selected drugs. The sodium hexametaphosphate anion crosslinker together with microspheres are more hydrophobic. They are also more compact in size and shape. Optimum loading at pH 3 was shown by sodium hexametaphosphate-anion-cross-linked microspheres. In contrast, at pH 4 optimum loading was shown by the sodium tripolyphosphate-anion-cross-linked microspheres. The sodium tripolyphosphate-anion-cross-linked microspheres are appropriate for the controlled release of rifampicin. While the sodium

hexametaphosphate-anion-cross-linked microspheres are suitable for the controlled release of hydroxy urea. Physically cross-linked chitosan microspheres' characteristics of drug release can be explained with respect to the encapsulated drugs with anion crosslinkers and the polymer matrices ionic interactions and their size. The loaded drugs of an initial burst release are Fickian in nature. They follow the first-order kinetics. However, drug release of the controlled step follows the zero order kinetic and revealed a non-Fickian nature.

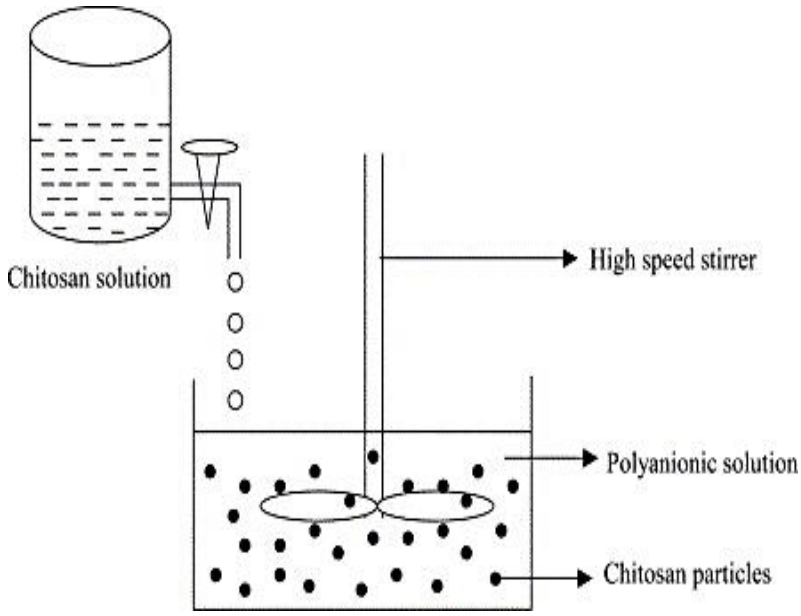


Figure 38. Diagram displayed the systems of chitosan microparticle preparaton by ionic gelation method (From: Agnihotri et al., 2004).

3.1.1.4 Spray drying method

The method of spray drying was reported to be suitable, easy, and reproducible to produce chitosan microspheres (Figure 39). When compare this technique with other encapsulation methods, found that this method is efficient to generate microspheres with the good distributions of narrow sized with low levels of

toxic residual organic solvent and small in particle sizes (Cevher *et al.*, 2006). The spray drying method has been reported to have many advantages. (1) It is using non-toxic solvents, mulsifiers or oils if a solvent is water. Therefore, it will leave no undesirable residuals toxic compound in final microparticle formulation. (2) The method is easily to scaled-up and can reach high in productivity. (3) This method is ideal to use for preparation of biomaterials for application of wound healing since the production of chitosan microparticle are in polydisperse systems (Vasconcellos *et al.*, 2011). Spray-drying method can also promote the microencapsulation. The obtained spray-dried hydrophilic microspheres are often water-swell able and porous making encapsulated active agent can be quick released (Mi *et al.*, 1999). Various applications are found in spray-drying method in a number of the chemical industries. For example in ceramics, organic chemicals, fertilizers, food, biological agents, polymeric resin, and pharmaceuticals. Hasçiçek *et al* (2003) prepared the microspheres of chitosan by the technique of spray drying for nasal administration having gentamicin sulfate as an active drug. High drug encapsulation efficiency is displayed, also good property of mucoadhesive appropriate for nasal administration, and suitable size of microspheres were observed. Desai and Park, 2005 created the process of chitosan–tripolyphosphate microsphere preparation through the method of spray-drying, by the usage of a model drug, acetaminophen. Microspheres of the chitosan–tripolyphosphate had a smooth surface and were spherical. Acetaminophen loaded in spherical chitosan–tripolyphosphate microparticles were found in the 3.1–10.1 mm size range. Spray-dried chitosan–tripolyphosphate microspheres were positively charged from +18.4 to +31.8 of zeta potential ranged. The microspheres' encapsulation efficiency was in 48.9–99.5% range. Chitosan–tripolyphosphate microspheres' drug release occurred through the diffusion pattern according to the Higuchi model.

Osman *et al* (2013) reported utilizing spray drying technique to prepare the ciprofloxacin microparticles for pulmonary administration. Ciprofloxacin was dissolved in dextran and chitosan solution and then the resulting solution was sprayed drying to produce microparticles. Ciprofloxacin microparticles showed improved aerosolisation properties and the encapsulated drug possessed high antimicrobial activity against two

of the common and resistant respiratory pathogens: *Pseudomonas aeruginosa* and *Staphylococus aureus*. The microparticles were also demonstrated to be safe on the lung epithelial cells.

Sander *et al* (2013) constructed bioadhesive chitosan microparticles containing metformin by spray drying aqueous dispersions of the drug in different molecular weights of chitosan. The result suggested that higher molecular weight of chitosan could improve bioadhesive properties of metformin microparticles. That could be due to a higher degree of interpenetration of mucosal residues and due to higher viscosities of the swelled particles.

Chitosan microparticle cross-linked by (TPP) tri-polyphosphate anions were produced by the method of spray drying (Kašpar *et al.*, 2013). *Ex-situ* cross-linking were employed by the two different cross-linking methods. In cross-linked chitosan nanoparticle, an aqueous chitosan solution has been spray-dried by the method of novel in-situ cross-linking and a two-fluid nozzle. A mixture of TPP and chitosan solution was fed to cross-linking occurred within individual droplets and three-fluid nozzle. Resulting microparticle morphology and the size have also been characterized and dependence on initial concentration of chitosan and the ratio with the cross-linking agent. Three-fluid nozzle that produced particles was observed to have good properties which were favorable in the terms of aqueous media stability. They allow chitosan concentration usage at higher value which can lead to appropriate applications for the microencapsulation.

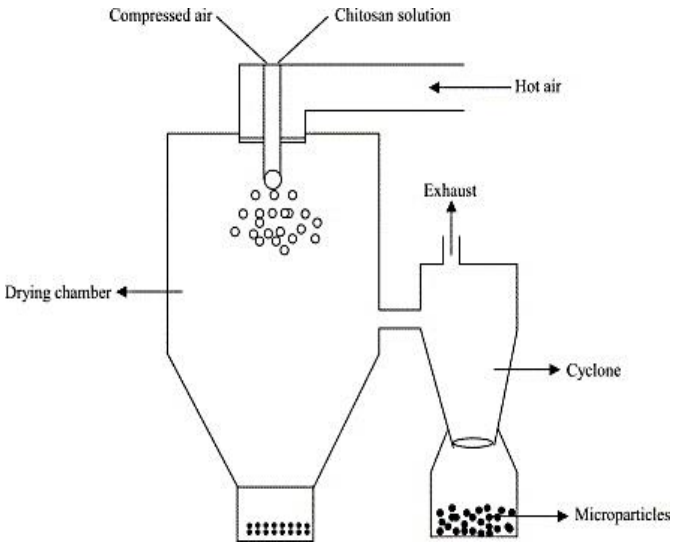


Figure 39. Scheme shows the chitosan microspheres preparation by spray drying method (From: Agnihotri et al., 2004).

3.1.2 Loading drugs to chitosan microspheres

Two methods of chitosan microspheres can be used for drug loading. The first method, drug loading can be done after (incubation) or particle formation and the second method, the drug can be loaded during the particle preparation (incorporation). Various methods of drug loading in chitosan microspheres have been developed to enhance the loading efficiency. It depends largely on the drug's physicochemical properties and on the preparation method. The result from previous reported works demonstrated that, incorporating the drug during particles formation can create maximum drug loading. However, it could be affected by parameter process such as an additive presence, the preparation method, and etc. Both water-insoluble and water-soluble drugs can be loaded into the systems of chitosan-based particles. Chitosan solution is mixed with the water soluble drugs to form homogeneous mixture. Then any of the discussed methods above can produce the particles.

Sodium diclofenac was loaded in high encapsulation efficiency upto 100% during the particles formation (González-Rodríguez *et al.*, 2002). Three model drugs having different pKa, including diclofenac sodium, salicylic acid and insulin were used

for the drug-chitosan nanoparticles or micro preparation by an ionic interaction. A high entrapment efficiency of micro/nanoparticles of an encapsulation of drug would be enhanced by increasing in the chitosan concentrations (Boonsongrit *et al.*, 2006).

3.1.3 The release of drug and kinetics of the chitosan microspheres

Drug release from chitosan-based particulate systems was found to depend on the density and size of particulate system, cross–linking extent, the morphology, drug physicochemical properties and also an adjuvant presence. The release of *In vitro* depends on polarity, pH, and enzymes presence in the dissolution media. The chitosan particulate system drug release involves three mechanisms. There are (a) the release from particle surface, (b) the diffusion from the matrix of swollen rubbery, and (c) the release according to the erosion of polymer. These mechanisms can be found in Figure 40.

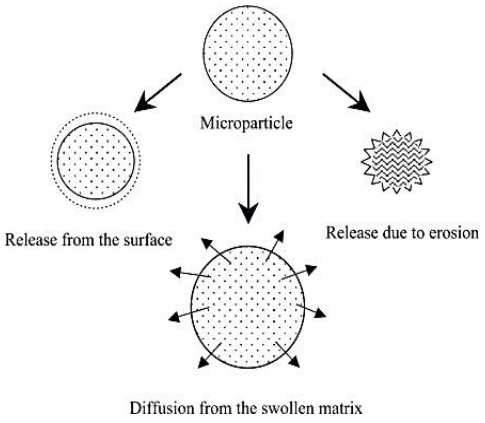


Figure 40. Picture shows the drug release mechanisms from the particulate systems (From: Agnihotri et al., 2004).

3.1.4 Chitosan particulate systems in pharmaceutical applications

3.1.4.1 Mucosal delivery

Mucosa is considered as the drug administration's potential sites. Drug delivery transmucosal routes such as rectal, nasal mucosal linings, vagina, oral cavity and ocular provide the distinct advantages over other delivery of drug system peroral administration. Advantages include possible first pass effect bypass, and pre systemic elimination avoidance of the gastro intestinal tract. They depends on particular drug (Sangeetha *et al.*, 2010). It is known that chitosan can increase the absorption of drug via mucosae without biological system damage. The chitosan action mechanism was considered to be a transient widening of epithelial cell tight junctions and the bio adhesion combination (Sonaje *et al.*, 2012). 4-Carboxybenzenesulfonamide-chitosan microspheres (4-CBS-chitosan) were prepared using electrospraying with acetazolamide as a model drug. 4-CBS-chitosan had 90% encapsulation efficiency for acetazolamide, forming 3.1 μm diameter microspheres with a low polydispersity index (0.4). Acetazolamide loaded 4-CBS-chitosan gave a sustained release of acetazolamide (～100% over 3 h) in gastric fluid stimulation (0.1 N HCl; pH 1.2), which was better than that obtained from acetazolamide-loaded chitosan (44% over 1.5 h). Thus, 4-CBS-chitosan microspheres are a possible drug carrier in acidic conditions, such as at the gastric mucosal wall (Suvannasara *et al.*, 2014).

There are many advantages of an oral vaccination over parenteral injection, however, low uptake in gastrointestinal tract lymphoid tissue and vaccine degradation in the gut still make oral vaccine development complicated (van der Lubben *et al.*, 2001). Both systemic and mucosal immune responses to prevent the disease transmission and were provided by the strategy of the mucosal vaccination. Vaccination via mucosal membranes requires the systems of delivery/adjuvants to increase antigen immunogenicity. Chitosan is a bio-adhesive, biocompatible, and biodegradable natural polysaccharide. It is a promising system for both as an adjuvant and the system of the delivery for mucosal vaccination (Günbeyaz *et al.*, 2010).

Chitosan microparticles were interacted with tripolyphosphate by the method of spray-drying loaded *pseudomonas exotoxin A*. The chitosan microparticles' mean particle sizes were from 1.09–1.46 μm in range and the efficiencies of antigen loading were higher than 59%. Microparticles had smooth surface and spherical in shape as the chitosan molecular weight was increased. The released *pseudomonas exotoxin A* from microparticles was found to be approximately about 60–80% (Taranejoo *et al.*, 2011).

3.1.4.2 Colon targeted drug delivery

The systems of colon-specific drug delivery have obtained an increasing importance in recent years. There are more effective in the local pathology treatment such as colorectal cancer, ulcerative colitis, and Chron's diseases. In addition, they also are more effective in conventional and labile molecules of the therapy system. An investigation of many natural polysaccharides for their potential application for drug delivery such as the systems of colon drug carrier have been considered and chitosan is an attractive polymer among others due to their biodegradability by colonic microflora, biocompatibility, nontoxicity, and the properties of mucoadhesion (Mennini *et al.*, 2012). Chitosan microspheres were composed by the method of emulsion cross-linking. Diltiazem hydrochloride active drug was encapsulated in the microspheres, following the coating of Eudragit S-100 by the technique of solvent evaporation. The chitosan microspheres were between 6.32 μm and 9.44 μm of a mean diameter. The microspheres were prepared by chitosan's emulsion cross-linking. The study of in vitro release of the non-coated chitosan microspheres in simulated GI or gastrointestinal fluid exhibited the pattern of a burst release in first hour. However, Eudragit S-100 coating microspheres allowed controlled release pattern of the diffusion fitting systems to the model of Higuchi. It was also appropriate for the delivery of colon-specific drug (Jose *et al.*, 2011).

3.1.4.3 Cancer therapy

Doxorubicin loaded chitosan microspheres have been prepared for many of the solid tumor treatments such as hepatocellular cancer, gastric, and bladder. Doxorubicin is known to have severe side effects when it administered at the high dose systematically which is similar to the most of an anticancer drugs. Microspheres from carboxymethyl cellulose and chitosan loaded Doxo or doxorubicin by an interaction of ion-exchange with groups of carboxyl in microspheres. The microspheres have size about 20±5 μm. The microspheres exhibited a noticeable behavior of pH-sensitivity with the Doxo accelerated release in an acidic environment according to the carboxylic groups' protonation in microspheres. The loaded chitosan microspheres containing Doxo revealed a sustained release manner in the phosphate buffered saline when compared to the non-resorbable commercial drug eluting beads. The release data was fitted to empirical relationship. It reveals the mechanism of a non-Fickian transport at n = 0.55–0.59. The result from this work showed that the bioresorbable microspheres are determining as the Doxo protential carriers (Weng *et al.*, 2011).

3.1.4.4 Topical delivery

The delivery of topical drug provides significant benefits for enhancing an effect of therapeutic and reducing in the administered compounds systemic side effects. Biopolymeric material-based system utilization can also play an important role in their applications and their new topical dosage form development (Bansal *et al.*, 2011, Sezer and Cevher, 2012).

Vitamin E incorporated chitosan microspheres for topical application were prepared using spray-drying method. Vitamin E which are normally used in cosmetic products has limited according to its low stability. However, vitamin E was found to be incorporated successfully to chitosan microspheres as justified in the results. Chitosan microspheres revealed a burst release at the first 5 minute after application as according to the studies of an *in vitro* release. Nevertheless, the release was lasted for

6 h. *In vivo* studies indicated that chitosan microspheres having Vitamin E is a promising formulation for an effect of the anti-aging since an increase in the skin elasticity and skin moisture, and the decrease in the skin roughness (Yenilmez *et al.*, 2011).

Insulin is an effective agent known to be effective for the patients treatment with type I diabetes, however, three or four injections are needed daily for best control of diabetes which definitely affect patient compliance (Elsayed *et al.*, 2014). Chitosan microspheres have been developed as a topical delivery vehicle. It is capable of bioactive insulin releasing therapeutic levels for many weeks with potential to sustain and stimulate the healing. Microspheres revealed that they can release bioactive insulin up to 25 days and also can be stabilizing. These are done by encapsulating insulin's crystalline form within the poly(*D*,*L*-lactide-co-glycolide) microspheres (Hrynyk *et al.*, 2010).

From the previous reviewed found that chitosan microspheres are promising drug delivery system that can be applied in many routes of application. Several methods have been reported in chitosan microspheres preparation including using cross-linking agent and spray drying technique. A number of cross-linking agents have been reported in chitosan preparation process. However, none of any report was utilized epoxidized natural rubber as a cross-linking agent in chitosan microspheres preparation. From previous chapter, ENR was previously removed any remaining proteins and successfully utilized for chitosan film preparation. In this chapter, dP-ENRs are subjected to be used as cross-linking agent in chitosan microspheres preparation. Spray drying technique was selected to be used for microspheres preparation.

3.2 OBJECTIVES

3.2.1 To find the optimum conditions for the preparation of chitosan microspheres by cross-linking reaction using deproteinated epoxidized natural rubber.

3.2.2 Characterization of physiochemical properties of the chitosan microspheres (with drug and without drug)

3.2.3 To evaluate the drug loading and the *in vitro* release of the model drug from the microspheres.

3.2.4 To evaluate the stability of the model drug.

3.3 EXPERIMENTAL

3.3.1 Chemicals

The reagents and substances used in the study are listed as following:

- Acetic acid, analytical grade (Labscan Ltd., Bangkok, Thailand)
- Alginate, analytical grade (Labscan Ltd., Bangkok, Thailand)
- Chitosan (mw = 300,000 and %degree of deacetylation = 80) (Seafresh Chitosan (Lab) Company Limited, Thailand)
- Diclofenac (raw material, China)
- Epoxidized natural rubber, 25 and 50 mole% epoxidation levels (ENR-25 and ENR-50) (Muang Mai Guthrie Co. Ltd., Thailand)
- Ethylene glycol diglycidyl ether (TCI America, USA)
- Methanol, analytical grade (Labscan Ltd., Bangkok, Thailand)
- Polyethylene glycol 200 (Merck®, Germany)
- Potassium dihydrogen phosphate, analytical grade (Ajax Finechem Pty Ltd., Australia)
- Sodium chloride, analytical grade (Labscan Ltd., Bangkok, Thailand)
- Sodium dihydrogen phosphate dihydrate, analytical grade (Guangdong, China)
- Sodium hydroxide, analytical grade (Labscan Ltd., Bangkok, Thailand)
- Sodium tripolyphosphate, analytical grade (Labscan Ltd., Bangkok, Thailand)
- Tetrahydrofuran, analytical grade (Labscan Ltd., Bangkok, Thailand)
- Water was purified in a Milli-Q system (Millipore, Bedford, MA, USA.

3.3.2 Instruments and equipments

- Analytical balance (Sartorious® model BSA 2248, scientific promotion co., Ltd., Thailand)

- Chromatographic Column (C8 reverse-phase column, 150 mm x 4.6 mm; particle size 5 µm; ACE®, Scotland)

- Differential scanning calorimeter (DSC7, Perkin Elmer, USA)

- Fourier transforms infrared spectrometer (spectrum one FT-IR spectrometer, Perkin Elmer, USA)

- High performance liquid chromatography (Agilent 1100 series, Germany)

- Hot plate and magnetic stirrer (Cemarec®2 Thermolyne SP46920-33, USA)

- Micropipette 1000 and 200 µL (Biorad®, USA)

- Modified Franze diffusion cell (model 57-951-061, Hanson Research Corporation, USA)

- Nuclear Magnetic Resonance (Fourier transform NMR spectrometer 500 MHz, Unity Inova, Varian, Germany)

- pH meter (Seven Easy S-20, USA)

- Spray Dryer (Mini Spray Dryer B-290, BÜCHI, Switzerland)

- Ultrasonic bath (model DSC 104, D.S.C. group co., Ltd., Thailand)

- UV-visible spectrophotometer (Hewlett Packard 8452A, Diode Array Spectrophotometer, USA)

- X-ray diffractometer (X'Pert MPD, Philips analytical, Netherlands

3.4 METHODS

3.4.1 Optimization for a suitable protocol to prepare chitosan microspheres

3.4.1.1 Optimization for a suitable concentrations of epoxidized natural rubbers in chitosan microspheres preparation.

The determination for an optimal epoxidized natural rubber concentrations in chitosan microspheres preparation, different formulations of chitosan microspheres were prepared with varying concentrations of dP-ENR-25 or dP-ENR-50 (0.10, 0.25, 0.50, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/100 mL) in tetrahydrofuran. Chitosan microspheres were prepared by dissolving chitosan 1.50 g in 1.0 % acetic acid solution (100 mL) at room temperature (30±1 °C) with magnetic stirrer for 24 h. Solution of dP-ENR-25 or dP-ENR-50 (1.0 mL) were added to the chitosan solution and stirred at room temperature (30±1 °C) for another 24 h. The optimal concentrations of dP-ENRs were selected from the resulting solution after 24 h stirring that contain the highest concentration of dP-ENRs and can still preserve clear solution without any precipitation or aggregation was observed.

3.4.1.2 Optimization for a suitable volume of epoxidized natural rubbers in chitosan microspheres preparation

The optimization of suitable volume of dP-ENRs as cross-linkers, the chitosan microspheres were prepared by dissolving chitosan 1.50 g in 1.0 % acetic acid solution (100 mL) at room temperature (30±1 °C) with magnetic stirrer for 24 h. Solution of 1.0 % dP-ENR-50 or 4.0 % dP-ENR-25 in varied volumes at 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 2.00, 2.50 and 3.00 mL in tetrahydrofuran was added to the chitosan solution and stirred at room temperature for another 24 h. The suitable volumes of dP-ENRs were chosen from the maximum volume that can give clears solution.

3.4.1.3 Optimization for a suitable concentration of chitosan solution for chitosan microspheres preparation.

Chitosan microspheres were prepared by dissolving different amount of chitosan (1.0, 1.50, 2.0 and 2.50 g) in 1.0 % acetic acid solution (100 mL) at room temperature (30±1 °C) with magnetic stirrer for 24 h. Solution of 1.0 % dP-ENR-50 in tetrahydrofuran (1.0 mL) was added to the chitosan solution. Aqueous solution of 1%TPP (1.0 mL) was added dropwise to the chitosan solution while stirring and continued stirring at room temperature for another 24 h. The resulting mixture was then spray dried (Mini Spray Dryer B-290, BÜCHI, Switzerland, Figure 41). Spray-drying conditions such as nozzle (500 µm), inlet temperature, liquid flow, and compressed spray air flow (represented as the volume of the drying air input) were set at 175°C, 2 mL/min, 1.2 m^3/min, respectively. Optimization was done on the basis of average particle size of chitosan microspheres which were determined by using laser particle analyzer (LPSA, Beckman coulter LS230, USA).

Figure 41. Spray Dryer (Mini Spray Dryer B-290, BÜCHI, Switzerland) (Picture from www.buchi.co.th).

3.4.1.4 Optimization for the optimal amount of diclofenac loading in the chitosan microspheres preparation

In this determination, diclofenac was prior dissolved in 5 mL methanol and added to 1.50% w/v of chitosan solution in 1% acetic acid (100 mL) at room temperature (30±1 °C) and continued sirring with magnetic stirrer for 24 h. Solution of 1% dP-ENR-50 in tetrahydrofuran (1 mL) was added to the chitosan solution. Aqueous solution of 1% TPP (1 mL) was added dropwise to the chitosan solution during stirring at room temperature (30±1 °C) and continued stirring for another 24 h. The amount of diclofenac was varied from 5, 10, 20, 25, 30, 40 and 50 mg which was previously dissolved in 5 mL methanol before adding to the chitosan solution. The resulting mixture was then spray dried. Spray-drying conditions such as nozzle (500 µm), inlet temperature, liquid flow, and compressed spray air flow (represented as the volume of the drying air input) were set at 175°C, 2 mL/min, 1.2 m^3/min, respectively. Optimization was done on the basis of any concentration that still give clear solution prior to spray drying and average particle size of chitosan microspheres which were determined by using laser particle analyzer (LPSA, Beckman coulter LS230, USA).

3.4.1.5 Optimization for a suitable concentration of tripolyphosphates (TPP) solution for microspheres preparation

In this investigation, the chitosan microspheres were prepared by dissolving 1.5 g of chitosan in 100 mL of 1% acetic acid solution. Solution of 1% dP-ENR-50 in tetrahydrofuran (1.0 mL) was added to the chitosan solution and stirred at room temperature (30±1 °C) for 24 h. Diclofenac (20 mg) in 5 mL methanol was added to the chitosan solution and stirred at room temperature (30±1 °C) for another 24 h. Aqueous solution of TPP (1.0 mL) at different concentrations (1%, 2%, 4%, 5% and 8% w/v) was added dropwise to the solution and stirred at room temperature (30±1 °C) for 24 h. The resulting mixture was then spray dried. Spray-drying conditions such as

nozzle (500 μm), inlet temperature, liquid flow, and compressed spray air flow (represented as the volume of the drying air input) were set at 175°C, 2 mL/min, 1.2 m^3/min, respectively. Optimization was done on the basis of average particle size of chitosan microspheres which were determined by using laser particle analyzer (LPSA, Beckman coulter LS230, USA).

3.4.2 Chitosan microspheres preparation

3.4.2.1 Preparation of blank chitosan microspheres without cross-linker.

Blank chitosan microspheres without cross-linker were prepared by dissolving chitosan (1.5 g) in 100 mL of 1% acetic acid solution. Aqueous solution of 1% w/v tripolyphosphates (1.0 mL) was then added dropwise to the chitosan solution and keep stirring at room temperature (30±1 °C) for another 24 h. The resulting mixture was then spray dried. Spray-drying conditions such as nozzle (500 μm), inlet temperature, liquid flow, and compressed spray air flow (represented as the volume of the drying air input) were set at 175°C, 2 mL/min, 1.2 m^3/min, respectively.

3.4.2.2 Preparation of blank chitosan microspheres cross-linked with dP-ENRs.

Blank chitosan microsphere cross-linked with dP-ENRs were prepared by dissolving chitosan 1.5 g in 100 mL of 1% acetic acid solution at room temperature (30±1 °C) with magnetic stirrer for 24 h. Solution of 4% dP-ENR-25 or 1% dP-ENR-50 in tetrahydrofuran (1 mL) was added to the chitosan solution and continued stirring at room temperature (30±1 °C) for another 24 h. Aqueous solution of 1% w/v TPP (1.0 mL) was added dropwise to the chitosan solution while stirring and the resulting solution was kept stirring for another 24 h. The resulting mixture was then spray dried.

Spray-drying conditions such as nozzle (500 μm), inlet temperature, liquid flow, and compressed spray air flow (represented as the volume of the drying air input) were set at 175°C, 2 mL/min, 1.2 m^3/min, respectively.

3.4.2.3 Preparation of chitosan microspheres cross-linked with dP-ENRs containing diclofenac.

Chitosan microspheres cross-linked with dP-ENRs containing diclofenac were prepared by dissolving chitosan 1.5 g in 100 mL of 1% acetic acid at room temperature (30±1 °C) with magnetic stirring for 24 h. Solution of 4% dP-ENR-25 or 1% dP-ENR-50 in tetrahydrofuran (1 mL) was added to the chitosan solution and continued stirring at room temperature (30±1 °C) for 24 h. Diclofenac, 20 mg in 5 mL methanol was then added to the chitosan solution and stirred at room temperature (30±1 °C) for another 24 h. Aqueous solution of 1% w/v tripolyphosphates (1.0 mL) were added dropwise to the solution and stirred at room temperature for 24 h. The resulting mixture was then spray dried. Spray-drying conditions such as nozzle (500 μm), inlet temperature, liquid flow, and compressed spray air flow (represented as the volume of the drying air input) were set at 175°C, 2 mL/min, 1.2 m^3/min, respectively.

3.4.3 Physicochemical characterization

3.4.3.1 Morphology observations

The surface morphologies of all the obtained chitosan microspheres were examined using scanning electron microscopy (SEM, Quanta400, FEI, Czech Republic, Figure 42). Prior to observation, samples were mounted on metal grids, using double-sided adhesive tape, and coated by gold under vacuum before observation.

Figure 42. Scanning Electron Microscope (SEM, Quanta 400, FEI, Czech Republic) (Picture from http://lexi.eng.uci.edu/facilities.html.).

3.4.3.2 Particle size distribution and polydispersity index (PI)

The particle size distributions and PI of all the obtained microspheres were analyzed using a laser particle analyzer (LPSA, Beckman coulter LS230, USA, Figure 43). The samples were suspended in butanol prior to determination.

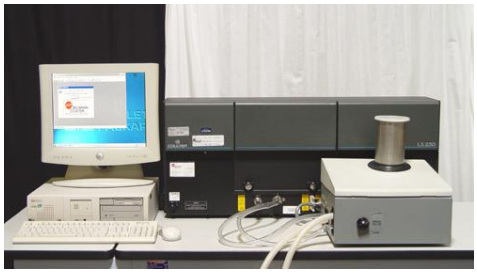


Figure 43. Laser particle analyzer (LPSA, Beckman coulter LS230, USA) (Picture from http://www.used-line.com/).

3.4.3.3 Zeta potential measurement

The zeta potential was calculated from the mean electrophoretic mobility value, which was determined by zeta potential analyzer (zetaPALS, brookhaven, USA, Figure 44). The microspheres cross-linked with dP-ENR-25 or dP-ENR-50 without drug was diluted with DI water before using for the determination.

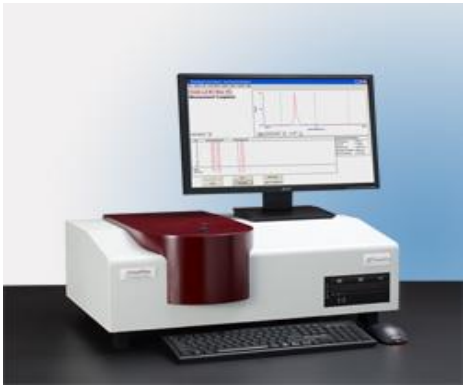


Figure 44. Zeta potential analyzer (zetaPALS, brookhaven, USA) (Picture from http:// http://www.brookhaveninstruments.com).

3.4.3.4 Differential scanning calorimetry (DSC)

The thermograms of pure diclofenac and chitosan microspheres cross-linked with dP-ENR-25 or dP-ENR-50 without drug and with drug were obtained using DSC7, Perkin Elmer, USA, Figure 45. The sample of 2–4 mg was accurately weighed into an aluminum pan with cover sealed. The measurements were performed under nitrogen purge over 20–500 °C at a heating rate of 10 °C/min.

Figure 45. Differential Scanning Calorimeter (Perkin Elmer DSC7, USA) (Picture from www.bidservice.com.).

3.4.3.5 X-ray diffraction

The physical state of pure diclofenac and diclofenac in the chitosan microspheres was assessed by XRD studies. X-ray powder diffraction spectra of pure diclofenac, chitosan microsphere were obtained at room temperature using a X-ray diffractometer (X'pert MPD, Philips, Netherlands, Figure. 46), with Co as anode material

and graphite monochromator, operated at a voltage of 40 kV. The sample were analyzed in the 2θ angle range 5-90° and the process parameters were set as step size of 0.05° (θ), scan step time of 1.0 sec.

Figure 46. Powder X-ray diffractometer (Philips X'Pert MPD, Netherland) (Picture from http://www.sseau.unsw.edu.au/xrd/xrdlab.htm).

3.4.3.6 FTIR spectroscopy

The Fourier Transform infrared (FTIR) spectra of pure diclofenac and all the obtained chitosan microspheres were recorded in an IR spectrometer (spectrum one FT-IR spectrometer, Perkin Elmer, USA, Figure. 47) in the wavenumber range 4000–400 cm^{-1} by accumulation of 16 scans at 4 cm^{-1} resolutions (KBr disks pressed under 10.0 tonnes pressure).

Figure 47. FT-IR spectrometer (Spectrum One, Perkin Elmer Ltd., UK).

3.4.4 Determination of drug content

The chitosan microspheres containing diclofenac were accurately weighed and placed in 50 mL methanol in a 100 mL erlenmeyer flask and sonicated

for 60 min and left standing at room temperature (30±1 °C) for 60 min. The clear supernatant was taken to determine the amount of diclofenac by using UV spectrophotometer (Hewlett Packard, 8452A) ($\boldsymbol{\lambda}$ = 278 nm). The drug content (%) were calculated by interpretation the concentration of samples to that of standard solutions of diclofenac (5-25 µg/mL), using the following equations (Jain *et al*., 2011).

$$\text{Drug content}(\%) = \frac{\text{Actual drug content}}{\text{Amount of drug added in each formulation}} \times 100$$

3.4.5 *In vitro* release studies

The USP 32 Type-1 basket dissolution test apparatus (USP 32 & NF27, 2009) was used to study the *In vitro* drug release. Phosphate buffer (0.2 M, pH 5.5, 900 mL) at 37.5±0.5 °C rotated at 100 rpm was used as the dissolution medium. Chitosan microspheres (100 mg) were placed in the dissolution medium. Sample (5 mL) were withdrawn at pre-determined time intervals (0, 15, 30, 45, 60, 90, 120, 150, 180, 210 and 240 min) and replaced with equal volumes of the dissolution medium. Sample were filtered through 0.45 µm. The test solution was analyzed for the drug release by using UV spectrophotometer (Hewlett Packard, 8452A) at 278 nm. All the experiments were done in triplicates. In the model-dependent approach, various kinetic models were applied to the release profiles, as in Eqs 1 – 5.

First order:	$\ln M_t = \ln M_o + K_1 t$	..... (1)
Higuchi:	$M_t = M_o + K_H t^{1/2}$	..... (2)
Hixson-Crowell:	$M_o^3 - M^{t3} = K_{HC} t$	..... (3)
Baker-Lonsdale:	$\frac{3}{2}[1 - (1 - \frac{M_t}{M_\alpha})^{2/3}] \frac{M_t}{M_\alpha} = k_t$	..... (4)
Korsmeyer-Peppas:	$\frac{M_t}{M_\alpha} = K_k t^n$	..... (5)

where, M_t is the cumulative amount of drug released at any specified time point and M_o is the initial amount of drug in the formulation. K_1, K_H, K_{HC} and K_k are rate constants for first order, Higuchi, HixsonCrowell and Korsmeyer-Peppas models, respectively. In Eq 5, $\frac{\mathbf{M_t}}{\mathbf{M_\alpha}}$ is the percentage of diclofenac released at time t and n is the release exponent that characterizes different release mechanisms (Dash *et al.*, 2010).

3.4.6 Stability study

The stability of the drug remaining during storage in the chitosan microspheres were performed using the method described by Cervera *et al*. (2004). The microspheres samples were stored in the desiccators at 40 $\pm$ 0.5°C/75% RH, 50$\pm$0.5°C/75% RH and 70$\pm$0.5°C/75% RH (RH = relative humidity) for 3 months (3 samples). The remaining amounts of diclofenac in each sample after storage at 1, 2 and 3 months were determined by using HPLC (Agilent 1100, diode array detector) method.

3.5 STATISTICAL ANALYSIS

Data were expressed as the mean$\pm$S.D. of at least three experiments. Analysis of variance (ANOVA) was used to test the statistical significance of differences among groups. The SPSS software package (SPSS 17.0 for Windows, SPSS Inc., Chicago, IL, USA). Comparison of means was performed by Duncan's test with confidence level as $P < 0.05$.

3.6 RESULT AND DISCUSSION

3.6.1 Optimization for a suitable formulation to prepare chitosan microspheres

In this study, we would like to prepare chitosan microspheres by cross-linking method. dP-ENR-25 and dP-ENR-50 were used as cross-linking agents. Diclofenac was used as a model drug. Tripolyphosphate was used as microsphere forming agent. To obtain a suitable formulation for chitosan microspheres preparation, various factors were optimized including chitosan concentrations, cross-linker concentrations, volume of cross-linking agent, drug concentration and tripolyphosphates concentration.

3.6.1.1 Optimization for a suitable concentration of epoxidized natural rubbers as cross-linking agent.

Deproteinated epoxidized natural rubber was used as a cross-linking agent in the microsphere preparation and moreover, since the microspheres were prepared by spray drying method, clear solution without any precipitation is required. Therefore, in the first optimization, the optimum concentration of dP-ENRs used for preparation process was studied. In the study 1.5% w/v of chitosan solution in 1% acetic acid was utilized. dP-ENR-50 and dP-ENR-25 in THF (1.0 mL) was added to the chitosan solution. The concentration of dP-ENRs were varied from 0.1 - 5% w/v. The optimum concentration was selected from the resulting mixed solution after stirring for 24 h and gave clear solution without any precipitation or aggregation was observed. The results (Table 22) demonstrated that clear solution were obtained after additional of 1 mL of dP-ENR-50 upto 1% w/v, whereas dP-ENR-25 can be added upto 4% w/v. Since we need the highest concentration in the preparation process, then, 1 mL of 1% w/v of dP-ENR-50 and 4% of dP-ENR-25 in THF were chosen for the microspheres preparation.

Table 22 Optimization result for a suitable concentration of epoxidized natural rubber for chitosan microspheres preparation.

dP-ENR-50 (w/v) in THF	Observation of the final solution after mixing	dP-ENR-25 (w/v) in THF	Observation of the final solution after mixing
0.1%	Clear solution	0.1%	Clear solution
0.25%	Clear solution	0.25%	Clear solution
0.5%	Clear solution	0.5%	Clear solution
1%	Clear solution	1%	Clear solution
2%	Aggregate	2%	Clear solution
3%	Aggregate	3%	Clear solution
4%	Aggregate	4%	Clear solution
5%	Aggregate	5%	Aggregate

3.6.1.2 Optimization for an optimum volume of epoxidized natural rubbers in microsphere preparation.

From the previous investigation, 4% w/v of dP-ENR-25 and 1% w/v of dP-ENR-50 were selected for microspheres preparation process. A suitable volume of both dP-ENRs was then studied. The investigation was performed by using 1.5% w/v of chitosan solution (100 mL) in 1% acetic acid. TPP solution (1% w/v, 1 mL) was added to the chitosan solution. The solution of 4% w/v dP-ENR-25 or 1% w/v dP-ENR-50 was added to the resulting solution in varying volumes from 0.25-1.5 mL and continued stirring for 24 h. The suitable volumes of dP-ENRs were selected from the resulting solution that still give clear solution without any precipitation or aggregation was observed. The results (Table 23) showed that both 4% w/v dP-ENR-25 and 1% w/v dP-ENR-50 can be added to the chitosan solution upto 1.0 mL which clear solutions were still observed after stirring for 24 h. Using the solutions of both dP-ENRs higher than 1.0 mL resulting in aggregation of excess dP-ENRs. It is therefore, 1 mL of both dP-ENRs was chosen for the microspheres preparation.

Table 23 Optimization for a suitable volume of 4% dP-ENR-25 and 1% dP-ENR-50 in THF for chitosan microspheres preparation.

4% w/v dP-ENR-25 (mL)	Observation of the final solution after mixing	1% w/v dP-ENR-50 (mL)	Observation of the final solution after mixing
0.25	Clear solution	Clear solution	0.25
0.50	Clear solution	Clear solution	0.50
0.75	Clear solution	Clear solution	0.75
1.00	Clear solution	Clear solution	1.00
1.25	Aggregate	Aggregate	1.25
1.50	Aggregate	Aggregate	1.50
2.00	Aggregate	Aggregate	2.00
2.50	Aggregate	Aggregate	2.50
3.00	Aggregate	Aggregate	3.00

3.6.1.3 Optimization for a suitable of chitosan solution for microspheres preparation.

A suitable concentration of chitosan (MW = 300 kDa) solution in 1% acetic acid was optimized for the microspheres preparation process. In this investigation 100 mL of chitosan solution in 1% acetic acid were used in varied concentrations from 1.0%, 1.5%, 2.0% and 2.5% w/v. Solutions of 1% dP-ENR-50 (1.0 mL) and 1% TPP (1.0 mL) were added to the chitosan solutions and subjected to spray drying after 24 h stirring. An optimum concentration of chitosan was selected based on their particle sizes. Figure 48 showed the particle sizes of the obtained microspheres by varying concentrations of chitosan solution in microsphere preparation process. Using chitosan solutions at 1.0% and 1.5% gave small particles in the same sizes (7.41-7.42 μm). Increasing concentrations of chitosan solution to 2% and 2.5%, larger particle were obtained in 8.30 and 10.03 μm, respectively. At high concentration of chitosan solution, larger sizes of particles were obtained that could be resulted form increasing

degree of cross-linking with dP-ENR. In this study 1.5% w/v of chitosan solution was selected for microsphere preparation, since using 1.5% w/v and 1% w/v particle sized were not significantly different.

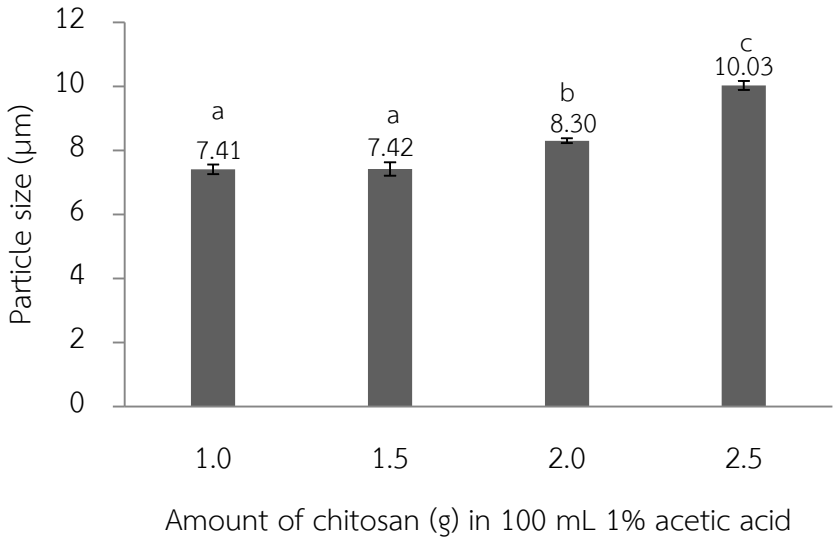


Figure 48. Graph demonstrates the particle sizes of the obtained chitosan microspheres by varying concentrations of chitosan solution in 1% acetic acid cross-linked with dP-ENR-50 without drug.

Different superscripts letters (a–c) within the same column indicate significant differences between formulations ($P < 0.05$). Analysis of variance (ANOVA) was used to test the statistical significance of differences among groups.

3.6.1.4 Optimization of for the optimal amount of diclofenac for chitosan microspheres preparation.

To determine a suitable amount of diclofenac that can be loaded to the chitosan microsphere, the microspheres were prepared by using 100 mL 1.5% w/v of chitosan solution in acetic acid cross-linked with 1% dP-ENR-50 (1.0 mL) before mixing with 1 mL of 1% w/v TPP solution. The different amount of diclofenac (5, 10,

20, 25, 30, 40 and 50 mg) in 5 mL methanol was added to the chitosan solution and the resulting solution was subjected to the spray drying. The selection criteria was based on the amount of diclofenac that still give clear solution prior to spray drying and the particle size of the obtained microspheres. The results from this determination revealed that when using diclofenac from 25 mg or above in the preparation process, the precipitate of diclofenac was observed in the final solution after stirring for 24 h. That could be due to the insufficient solvent to dissolve diclofenac when using high amount. The solutions with precipitate were not suitable for spray drying, therefore, they were not further utilized for spray drying process. In contrast to the formulations that used diclofenac at 5, 10 and 20 mg, clear solutions were achieved after 24 h stirring. Hence, these solutions were continued to spray drying process. The particle sizes of the obtained microspheres were determined and the results are displayed in Figure 49. The mean particle size of the microspheres obtained from the formulations using 5, 10 and 20 mg of diclofenac were 5.41, 6.03 and 6.43 μm, respectively. From the results, 20 mg of diclofenac was selected to be used in the microspheres preparation process. Even though, their particles sizes were bigger than the formulations using 5 and 10 mg of diclofenac, but still less than 10 μm, and we would like to prepare the microspheres that have high drug loading.

3.6.1.5 Optimization for a suitable concentration of tripolyphosphates (TPP) in microspheres preparation.

Tripolyphosphate (TPP) is one of the most important reagent in the chitosan microspheres preparation, since it plays roles as co-cross-linker as well as microspheres forming agent. It is therefore, investigation for a suitable amount of TPP for the microspheres preparation was performed. The microspheres were prepared by using 100 mL of 1.5% w/v of chitosan solution in acetic acid cross-linked with 1% dP-ENR-50 (1.0 mL). Diclofenac (20 mg) was previously dissolved in 5 mL methanol and then added to the chitosan solution and continued stirring. Different concentration of TPP solution in water (1%, 2%, 4%, 5% and 8%, 1 mL) was added to the resulting

solution and keep stirring for 24 h. The final solutions were subjected to spray drying. The optimization for a suitable concentration of TPP was selected on the basis of the particle sizes of the obtained microspheres. The particle sizes of the microspheres obtained from different formulations are summarized in Figure 50. The mean particle sizes of the chitosan microspheres obtained from the formulations using 1 mL of TPP at 1, 2, 4, 5 and 8% w/v were 6.76, 7.42, 7.42, 8.32 and 10.03 μm, respectively. The particle sizes was found to be increased upon increasing the concentration of TPP, could be due to the aggregation enhancement of nano- and microspheres by TPP to form bigger size. The lowest size (6.76 μm) was obtained when using 1mL of 1% w/v of TPP solution and the largest size was obtained when using 1 mL of 8% w/v of TPP solution. From these results, 1 mL of 1% w/v of TPP solution was selected to be used in the chitosan microsphere preparation process.

From all investigations, the optimum formation for chitosan microspheres preparation were using 100 mL of 1.5% w/v of chitosan solution in 1% acetic acid, 1 mL of 1% dP-ENR-50 or 1 mL of 4% dP-ENR-25, diclofenac 20 mg (1 5 mL methanol) and 1 ml of 1% w/v TPP solution (Table 24).

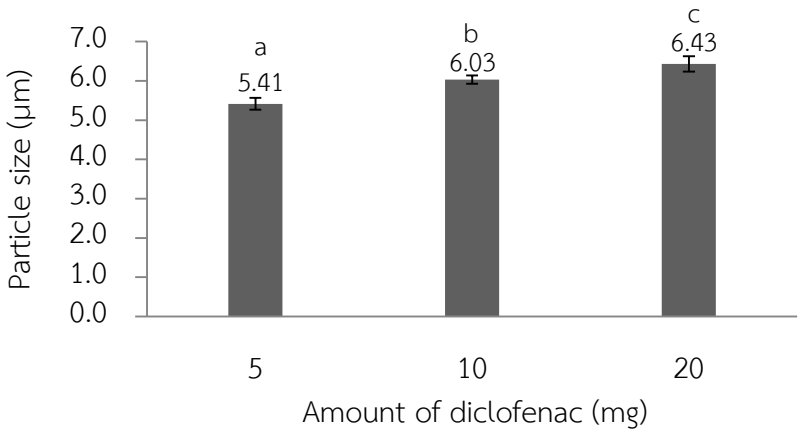


Figure 49. The particle sizes of chitosan microspheres obtained from the formulations using 5, 10 and 20 mg of diclofenac in the preparation process.

Different superscripts letters (a–c) within the same column indicate significant differences between formulations ($P < 0.05$). Analysis of variance (ANOVA) was used to test the statistical significance of differences among groups.

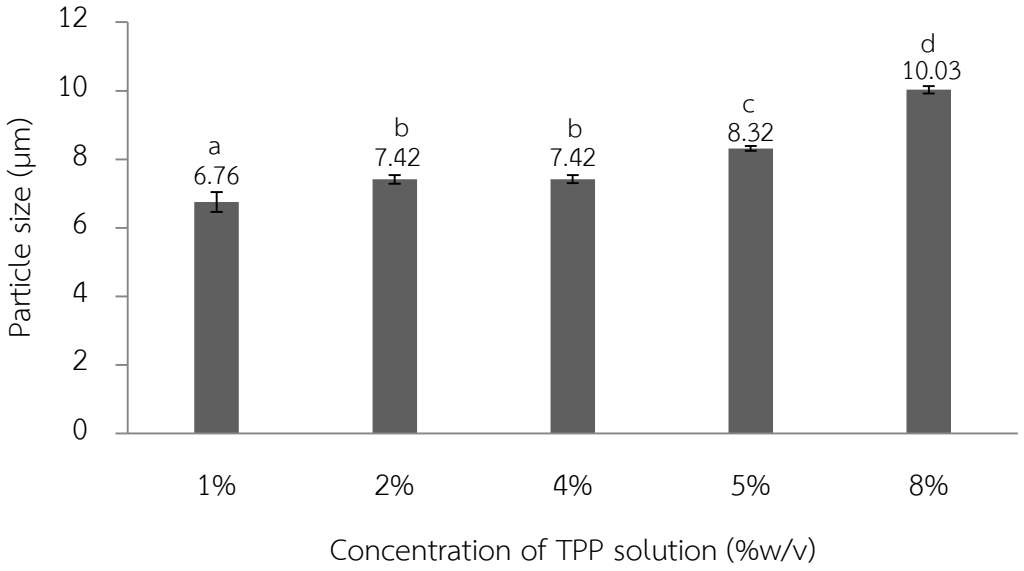


Figure 50. Particle sizes of the obtained chitosan microspheres using different concentrations of TPP solutions in preparation process.

Different superscripts letters (a–d) within the same column indicate significant differences between formulations ($P < 0.05$). Analysis of variance (ANOVA) was used to test the statistical significance of differences among groups.

Table 24 The optimum formulation for preparation of chitosan microspheres cross-linked with dP-ENR containing diclofenac.

Materials	Compositions
Chitosan (MW = 300kDa)	1.5g in 100 mL in 1% acetic acid
4% w/v dP-ENR-25	1 mL
1% w/v dP-ENR-50	1 mL
Diclofenac	20 mg
Methanol	5 mL
1% w/v Tripolyphosphate	1 mL

3.6.2 Physicochemical characterization

Chitosan microspheres cross-linked with dP-ENR-50 (CH-dP-ENR-50) and with dP-ENR-25 (CH-dP-ENR-25) containing diclofenac and without drug were obtained from the selected formulation (Table 24) after spray drying as light yellow powders (Figure 51 a-d). More intense yellow color was observed in the chitosan microspheres containing diclofenac than the microspheres without drug.

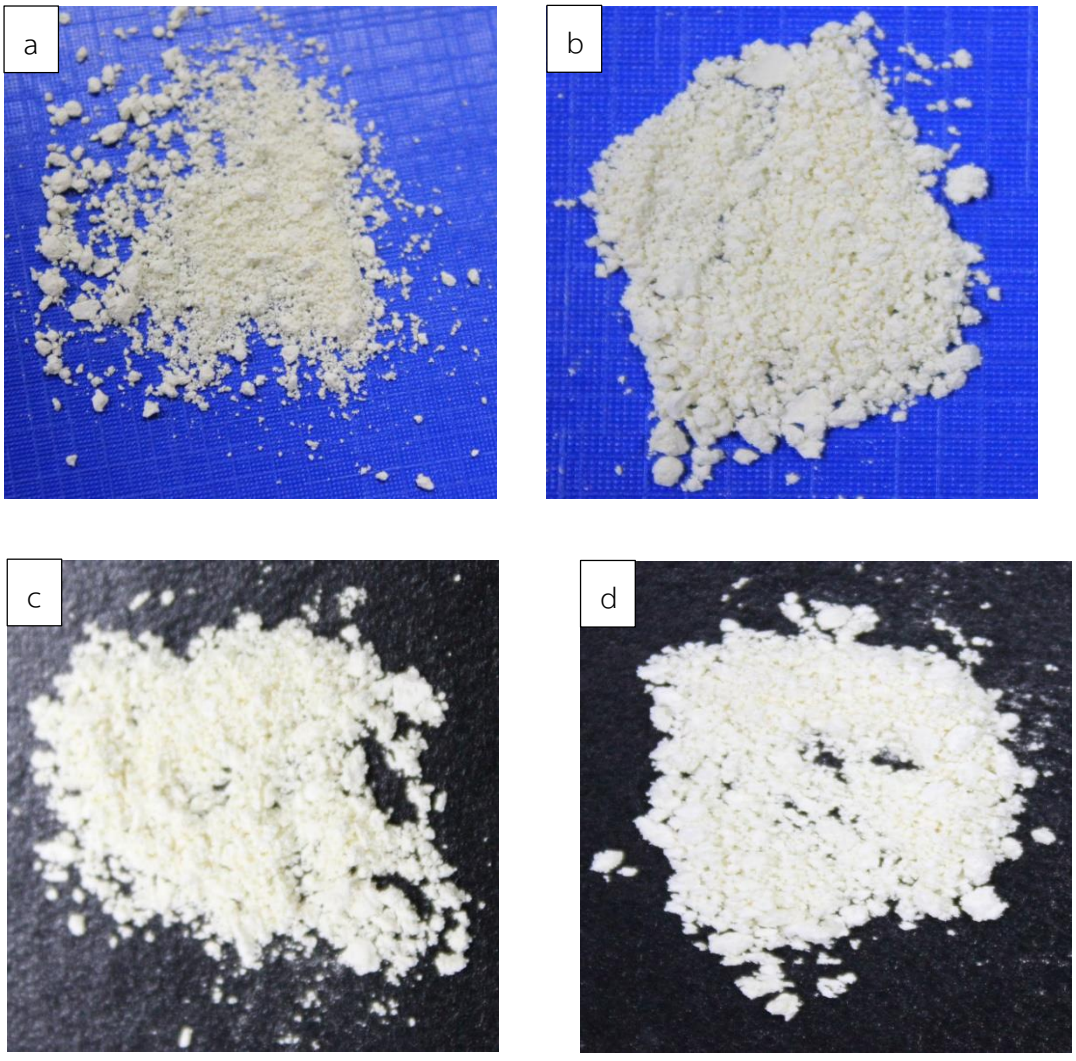


Figure 51. Picture of the obtain chitosan microspheres (a) CH-dP-ENR-50 without drug; (b) CH-dP-ENR-50 with drug; (c) CH-dP-ENR-25 without drug and (d) CH-dP-ENR-25 with drug.

3.6.2.1 Morphology observations

Microstructural properties of the obtained microspheres were determined by Scanning Electron Microscope (SEM) and the SEM micrographs are displayed in Figure 52. The SEM micrographs of chitosan microspheres cross-linked with both dP-ENRs without drug shown in figures 52a-d. The products were obtained in irregular shapes having various sizes upto about 300 μm when observed at 100x magnification (Figures 52a, c). However, when observe using 5,000 times magnification on the surface of each particle, nanospheres were noticed (Figures 50b, d). Those nanoparticles were spherical in shape and having size less than 1 μm. Therefore, the blank microparticles were formed by the accumulation of a number of nanospheres. The SEM micrographs of chitosan microspheres cross-linked with both dP-ENRs containing diclofenac are illustrated in Figures 50e-h. The observation was performed at 5,000x and 10,000x magnifications. The results revealed that products were obtained in spherical shapes both by using dP-ENR-25 and dP-ENR-50, having sizes ranging from nanosize upto 5 μm. The accumulations of a number of nano- and microspheres were observed in both dP-ENRs. Moreover, it is noticed that nanoparticles are deposited on the surface of microparticles. From SEM results indicated that the nanospheres and microspheres were successfully obtained by using the selected formulation. No crystal of diclofenac was presented in the obtained particles that could be due to the well disperse of the drug in amorphous form.

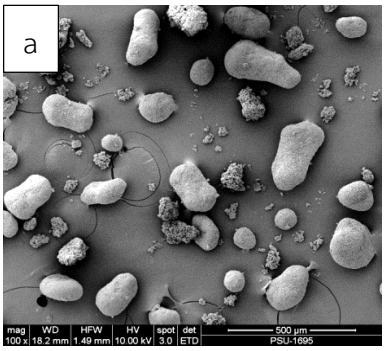
3.6.2.2 Particle size and Zeta potential measurement of the obtained chitosan microspheres.

The mean particle size (Table 25) of chitosan microspheres without cross-linker (CH-uncross-link) and cross-linked with dP-ENR-25 (CH-dP-ENR-25) and with dP-ENR-50 (CH-dP-ENR-50) were 8.24, 10.35 and 10.89 μm, respectively. Smaller size was observed from the formulation of CH-uncross-link than the cross-linked microspheres. Since, previously discussed (SEM) that the products were obtained ranging from nano-size upto about 5 μm which in contrast to the results from particle size analyzer. Larger

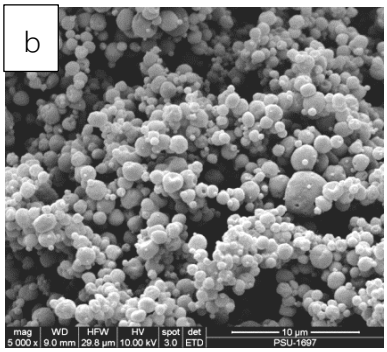
sizes by particle size analyzer could be to gross size measurement of the accumulated particles. The zeta potentials of chitosan microspheres without cross-linker and cross-linked with dP-ENR-25 and dP-ENR-50 were +38.42 ± 3.06, +29.74 ± 2.00 and +34.73±2.24, respectively. The positive zeta potentials of the microsphere could be due to the quaternary ammonium groups of chitosan. The results indicated that the degree of cross-linking reaction did not have any effect on the zeta potential of the microspheres.

3.6.2.3 Differential scanning calorimetry (DSC)

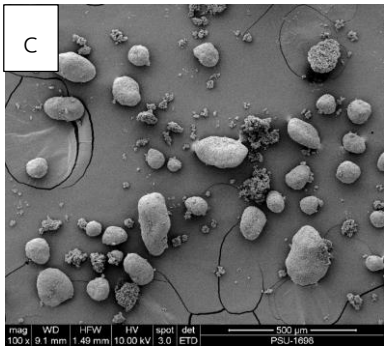
Differential scanning calorimetry is a thermal analysis technique used to measure changes in heat flows associated with material transition. DSC measurements could provide both qualitative and quantitative information on endothermic (heat absorption) and exothermic (heat evolving) process. DSC is commonly utilized to determine the glass transition temperature and crystalline melting point of polymeric material. In this study all samples including pure diclofenac and the obtained microspheres were subjected for DSC analysis. DSC thermogram of pure diclofenac shows a melting endotherm at about 172.6 °C (Figures 53a). The DSC thermogram of microspheres showed two peaks. The first broad endothermic peaks in a range of °C were due to water loss (Iturriaga *et al.*, 2014, Pereira Jr *et al.*, 2015). The second broad exothermic peaks in a range of could be due to the degradation of chitosan suggested by (Shivashankar and Mandal, 2013). In the case of drug-loaded microspheres, no peak corresponding to the melting endotherm of diclofenac, indicating that the amorphous dispersion of diclofenac in the polymer matrix.



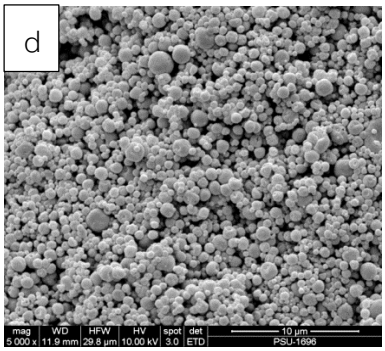
(a) CH-dP-ENR-50 without drug (x100)



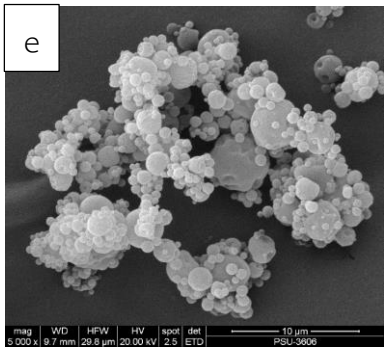
(b) CH-dP-ENR-50 without drug (x5000)



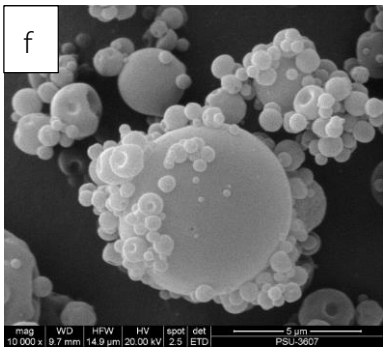
(c) CH-dP-ENR-25 without drug (x100)



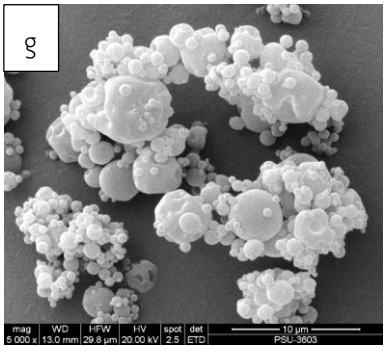
(d) CH-dP-ENR-25 without drug (x5000)



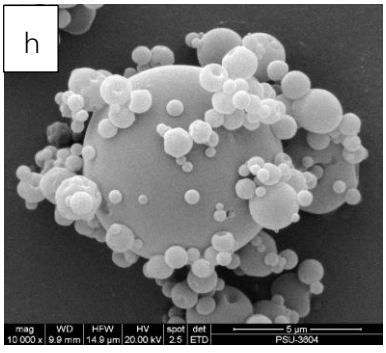
(e) CH-dP-ENR-50 with drug (x5000)



(f) CH-dP-ENR-50 without drug (x10000)



(g) CH-dP-ENR-25 with drug (x5000)



(h) CH-dP-ENR-50 with drug (x10000)

Figure 52. SEM micrographs of chitosan microspheres cross-linked with dP-ENRs both without and with drug at different magnifications

Table 25 Mean particle sizes and Zeta potential values of the obtained chitosan microspheres.

Formulation	Particle size (μm)	PI	Zeta potential (mV)
CH-uncross-linked	8.24	1.06	+38.42±3.06
CH-dP-ENR-25	10.35	0.92	+29.74±2.00
CH-dP-ENR-50	10.89	0.82	+34.73±2.24

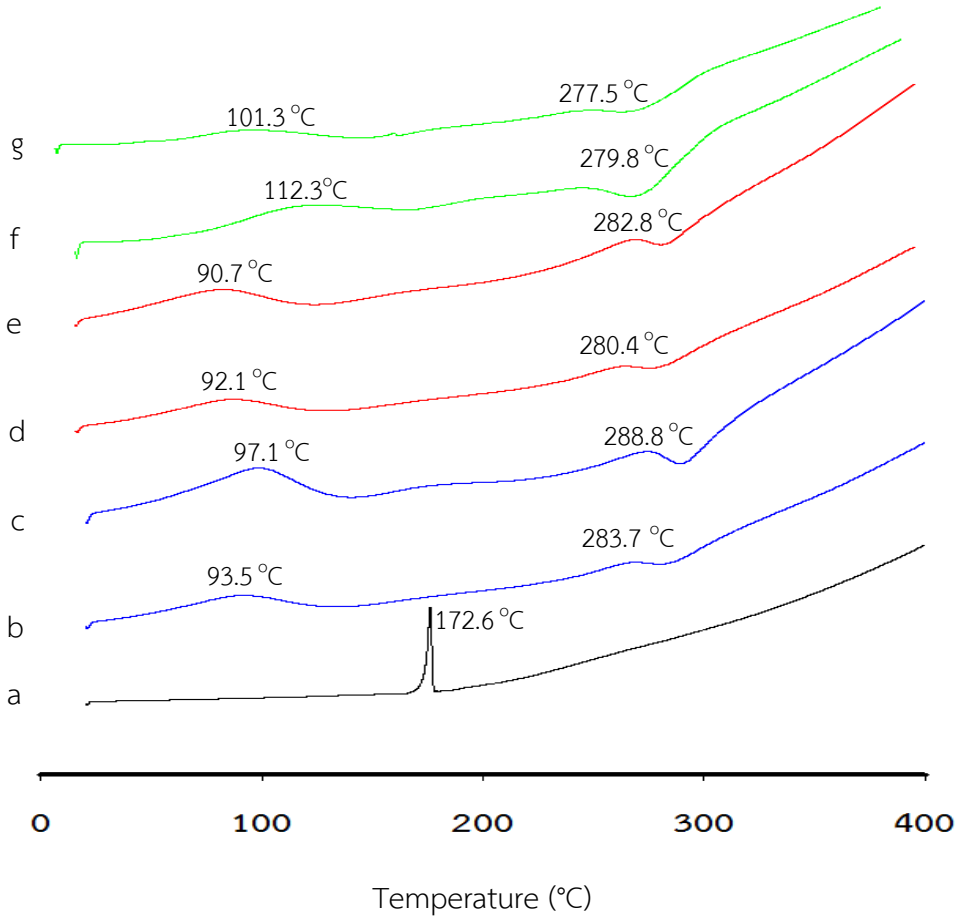


Figure 53. DSC thermograms, (a) Diclofenac; (b) chitosan microspheres cross-linked with dP-ENR-25 without drug; (c) chitosan microspheres cross-linked with dP-ENR-25 with drug; (d) chitosan microspheres cross-linked with dP-ENR-50 without drug; (e) chitosan microspheres cross-linked with dP-ENR-50 with drug; (f) chitosan microspheres uncross-linked without drug and (e) chitosan microspheres uncross-linked with drug.

3.6.2.4 X-ray diffraction

X-ray diffraction patterns are shown in Fig. 54 correspond to diclofenac (a), chitosan microspheres cross-linked with dP-ENR-25 without drug (b), chitosan microspheres cross-linked with dP-ENR-25 with drug (c), chitosan microspheres cross-linked with dP-ENR-50 without drug (d), chitosan microspheres cross-linked with dP-ENR-50 with drug (e), chitosan microspheres uncross-linked without drug (f) and chitosan microspheres uncross-linked with drug (e). The diffractogram of diclofenac powder (Fig. 54a) shows several sharp peaks indicated crystallinity property of the drug. The diffractogram for the chitosan microspheres uncross-linked and chitosan microspheres cross-linked with dP-ENR-25 or chitosan cross-linked with dP-ENR-50 with diclofenac (Fig. 54g, 54c, 54e) indicated that diclofenac presented in microspheres in amorphous form. The lost in degree of crystallinity is probably due to the non-uniform insertion of diclofenac within the chitosan polymer chains of the microspheres and the broad peak of chitosan microspheres may be due to the diclofenac reaction between chitosan, which may destroy the crystalline structure of chitosan (Hosseini *et al.*, 2015). Therefore, cross-linked microsphere matrix should be more suitable for controlled release applications that require slow release rates. Shivashankar and Mandal (2013) prepared chitosan microspheres cross-linked with glutaraldehyde loaded bupivacaine for buccal administration. XRD patterns indicated that bupivacaine peaks were not seen for the drug-loaded microspheres due to encapsulation of the drug in the polymeric network (Shivashankar and Mandal, 2013) and the drug was well dispersed in amorphous form.

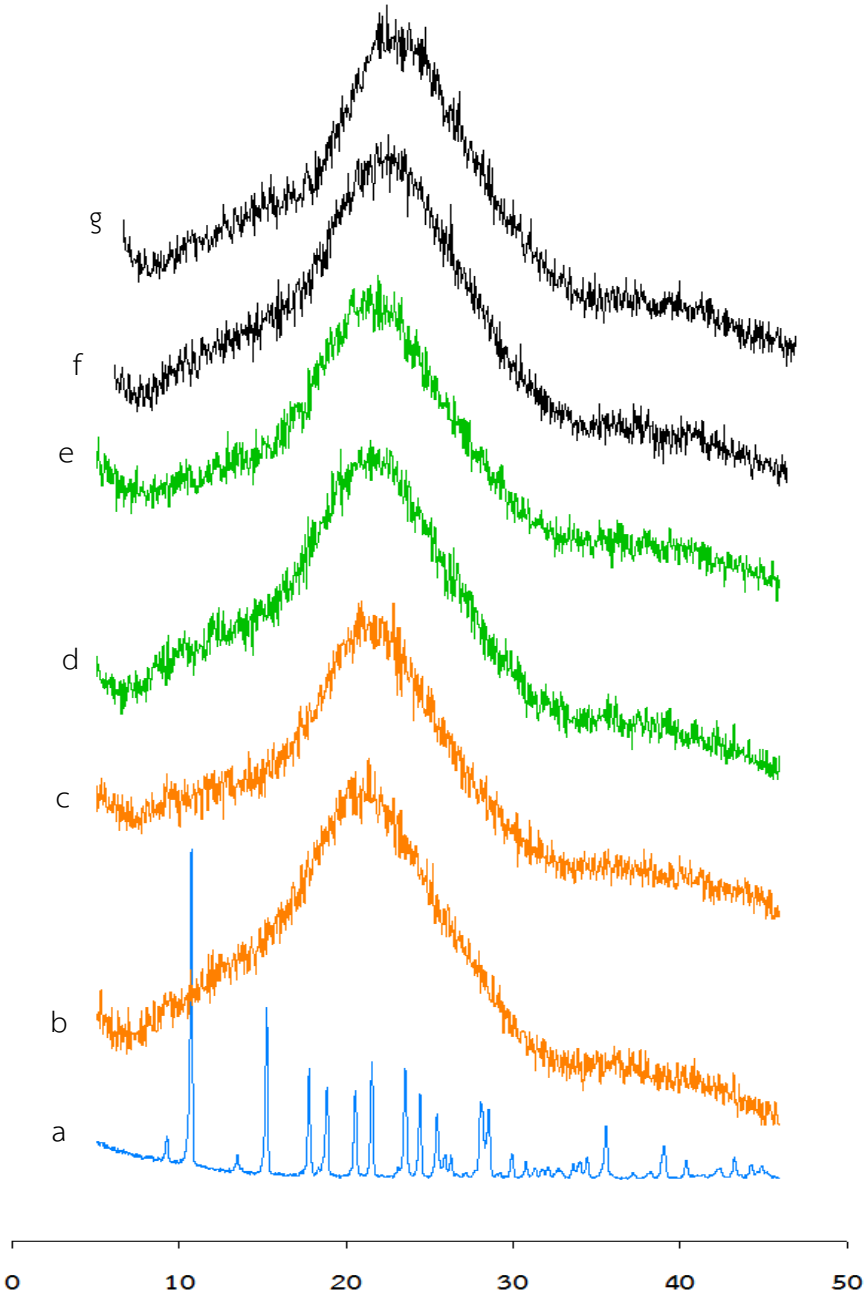
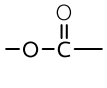


Figure 54. X-ray diffraction spectra of (a) Diclofenac; (b) chitosan microspheres cross-linked with dP-ENR-25 without drug; (c) chitosan microspheres cross-linked with dP-ENR-25 with drug; (d) chitosan microspheres cross-linked with dP-ENR-50 without drug; (e) chitosan microspheres cross-linked with dP-ENR-50 with drug; (f) chitosan

microspheres uncross-linked without drug and (e) chitosan microspheres uncross-linked with drug.

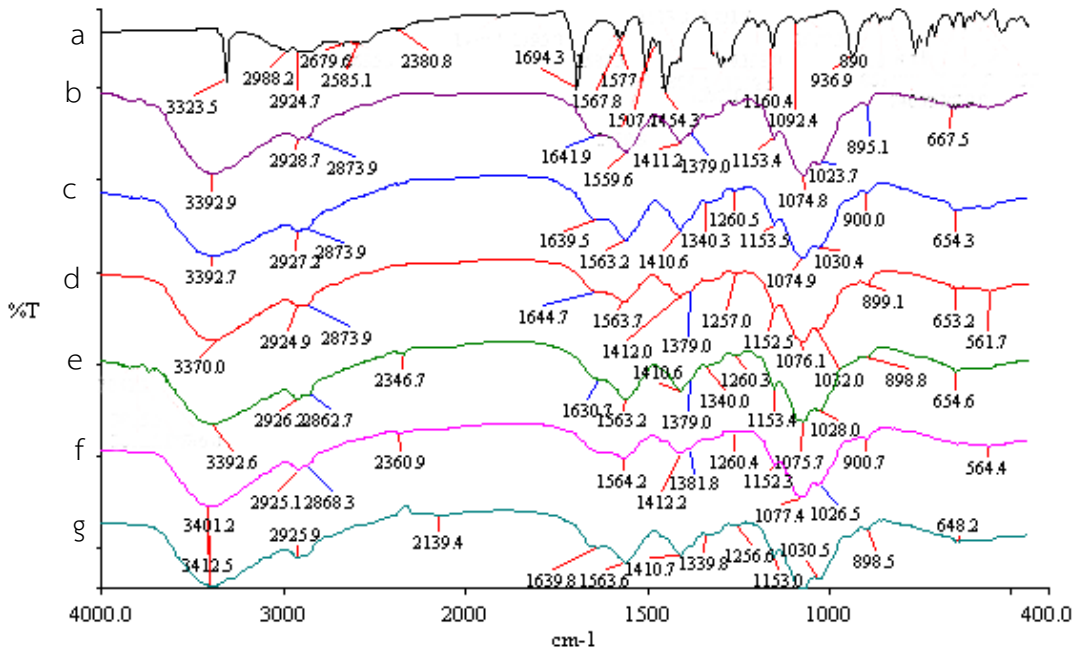
3.6.2.5 FTIR spectroscopy

To investigate the interaction of diclofenac with the microspheres compositions FT-IR was used. The FT-IR spectra of diclofenac, chitosan microspheres uncross-linked with and without drug, chitosan microspheres cross-linked with dP-ENRs with and without drug are displayed in Figure 55. FT-IR spectrum of pure diclofenac (Figure 55a) shows two important characteristic peaks at 3323 cm^{-1} and 1694 cm^{-1} which belong to –NH and –C=O stretching vibration, respectively. Moreover, peaks at 1587 cm^{-1}, 1577 cm^{-1} and 1507 cm^{-1} are the characteristic of –NH-ring stretching,  , and aromatic ring, respectively (Pawar *et al.*, 2013). FT-IR of all chitosan microspheres both uncross-linked and cross-linked with dP-ENRs and also with or without drug are shown in similar patterns. The main characteristic belongs to the characteristic of chitosan since it is the main composition in the formulation (Table 24). Chitosan microspheres both cross-linked with dP-ENRs (Figure 55e and g) and uncross-linked (Figure 55c) containing diclofenac show disappearance of both –NH stretching and carbonyl stretching characteristic of diclofenac and may be shifted to the lower frequencies. The reaction between those function groups of the drug with microspheres compositions would occur. The possible interactions could be due to the hydrogen bonding interactions (Misra *et al.*, 2010, Rasel and Hasan, 2012).

3.6.3 Drug entrapment efficiency of the obtained microspheres.

The chitosan microspheres were obtained by spray drying method of resulting solution after all compositions were mixed. It is therefore, the drug entrapment efficiencies of each obtained chitosan microspheres were assessed. The entrapped drug was extracted from the microspheres by using methanol and determined by UV-

Visible spectroscopy (λ = 278 nm) as described in an appendix. The drug entrapment efficiencies are summarized in Table 26. The drug entrapment efficiencies were calculated on the basis of the ability of the microspheres to entrap the model drug compared to the added amount before spray drying. The results revealed that slightly lost of the model drug was observed. The microspheres could entrap the model drug in a range of 92.24-94.71% w/w. Higher drug entrapment efficiencies were detected in the microspheres obtained from formulation using cross-linking agent than that without cross-link reaction. That could be due to the more efficiency in network formation in chitosan microspheres cross-linked with dP-ENRs resulting in better drug entrapment..



Wave number (cm^{-1})

Figure 55. FT-IR spectra of (a) diclofenac; (b) chitosan microspheres uncross-linked without drug; (c) chitosan microspheres uncross-linked with drug; (d) chitosan microspheres cross-linked with dP-ENR-25 without drug; (e) chitosan microsphere cross-linked with dP-ENR-25 with drug; (f) chitosan microspheres cross-linked with dP-ENR-50 without drug and (g) chitosan microspheres cross-linked with dP-ENR-50 with drug

Table 26 %drug entrapment efficiencies of diclofenac in microspheres.

Formulations	% drug entrapment in chitosan microspheres (%w/w) (mean±SD, n=3)
Chitosan microspheres uncross-linked	92.24±1.75
Chitosan microspheres cross-linked with dP-ENR-25	94.71±1.87
Chitosan microspheres cross-linked with dP-ENR-50	94.70±1.62

3.6.4 *In vitro* release studies

The release profiles of diclofenac from chitosan microspheres uncross-linked with drug and chitosan microsphere cross-linked with dP-ENR-25 or dP-ENR-50 containing diclofenac in phosphate buffer pH 5.5 at 37 ℃ are shown in Figure 56. Diclofenac loaded chitosan microspheres cross-linked with dP-ENRs showed an initial burst release of 19.71 % for dP-ENR-25 and 27.76% for dP-ENR-50, respectively, which may be accounted for the bioactive adsorbed on the surface. All of the diclofenac was released form chitosan microspheres uncross-linked within 90 min, whereas, both microspheres cross-linked with dP-ENR-50 and dP-ENR-50 gave similar release patterns, which show slower rate. They could release slowly form 15 min to 100% within 180 min. The slower release form microspheres prepared by cross-linking reaction with dP-ENRs cloud be due to the high cross-linked density. Therefore, the drug were penetrated out from the network slowly.

Kulkarni *et al*. (1999) recommended the release of the drug from the chitosan microspheres involves three different mechanisms: (a) release from the surface of particles, (b) diffusion through the swollen matrix, and (c) release due to polymer

erosion. An initial high release is observed due to the dissolution of surface adhered drug, whereas the drug release at the later time is due to the diffusion process, which is much slower when compared to the initial release (Kulkarni *et al.*, 2007).

He *et al*. (1999) reported prepared chitosan microspheres cross-linked with glutaraldehyde were prepared by a spray drying method. The release of model drugs (cimetidine, famotidine and nizatidine) from these microspheres was rapid at initial state, and accompanied by a burst effect. The amount of the drug released was slightly influenced by the amount of cross-linking agent. When the cross-linking density increased or the solubility of the drug decreased, the amount of the drug released was slightly reduced (He *et al.*, 1999).

Similarly, the release profile of diclofenac sodium from the chitosan microgranules loaded diclofenac sodium has also been studied in an acidic media (pH 2.0). The drug could release upto 85% of the entraped drug from microgranules in and acidic medium within 5 h (Gupta and Ravi Kumar, 2000).

Moreover, ambroxol hydrochloride was released from chitosan microspheres in sustained drug release pattern during 8 h of the study time. The drug was released from the developed microspheres at initial burst effect followed by slow diffusion of drug from microspheres matrix (Gangurde *et al.*, 2011).

3.6.5 Release kinetics

The release kinetic pattern from all chitosan microspheres were analyzed by using Sigma Plot fitting program (Version 12) and the results are illustrated in Table 27. The best fitting model was determined from regression analysis. The result indicated that the release kinetic of diclofenac from uncross-linked chitosan microspheres was followed Korsemeyer-peppas model in non Fickian diffusion pattern, indicated that the drug was released by combination of both diffusion and erosion control rate release. However, the release kinetic pattern of diclofenac from chitosan

microspheres cross-linked with both dP-ENRs were fitted with Hixon and Crowell. Therefore, the release of the drug could be due to the dissolution and the changes in surface area and diameter of the microspheres.

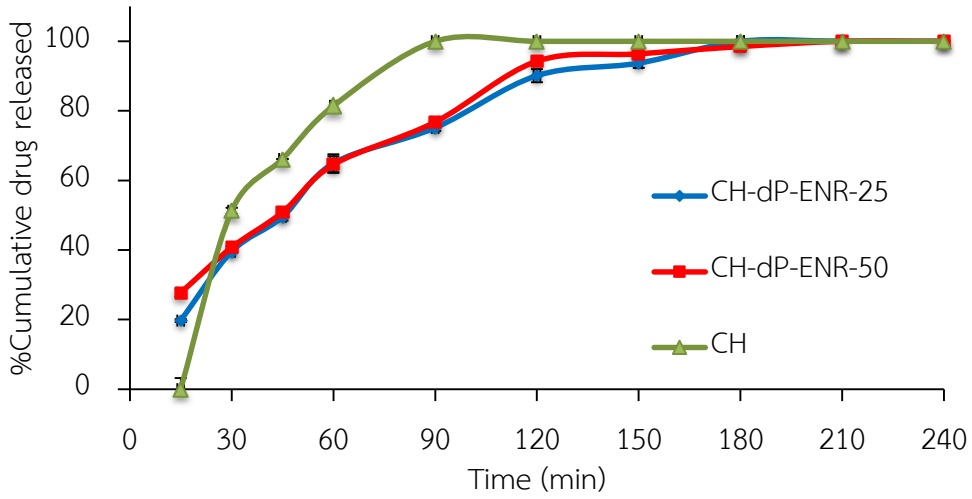


Figure 56. Release profile of diclofenac from the chitosan microspheres (n = 6).

CH-dP-ENR-50 = chitosan microspheres cross-linked with dP-ENR-50 containing diclofenac

CH-dP-ENR-25 = chitosan microspheres cross-linked with dP-ENR-25 containing diclofenac

CH = chitosan microspheres uncross-linked containing diclofenac

Table 27 Kinetics analysis of the release data of diclofenac from chitosan microspheres treated with kinetic equation models*

Formulation	First order		Hixon and Crowell		Higuchi model		Baker and Lonsdale			Korsemeyer-peppas model	
	r^2	k	r^2	k	r^2	k	r^2	k	r^2	K	n
ENR-25	0.9941	0.0168	0.9951	0.0046	0.9832	7.7062	0.9509	0.0017	0.9846	6.6036	0.5330
ENR-50	0.9897	0.0179	0.9899	0.0048	0.9757	7.6547	0.9660	0.0018	0.9794	9.5994	0.4531
Uncross-linked	0.9037	0.0228	0.9297	0.0065	0.8587	9.5984	0.8009	0.0022	0.9325	2.1814	0.8655

*****Sigma Plot v.12 was used for the data treatment

3.6.6 Stability of diclofenac in the obtained chitosan microspheres.

The stability of diclofenac in microspheres formulation were determined by storage the microspheres samples in climatic stability chamber at 40 ºC, 50 ºC and 70 ºC in 75% relative humidity for 90 days. The stability profiles of diclofenac in microspheres samples at different storage conditions are shown in Figures 57-59. At 40 ºC (75% RH) (Figure 57), slowly during 90 days of storage time. The degradation was found to be in similar patterns for all microspheres either cross-linked with dP-ENRs or uncross-link. Only 3% drug lost were observed. Therefore, dP-ENR which was used as cross-linker did not have any effect on the entrapped drug. Faster degradation of diclofenac was noticed when the microspheres were kept at 50 ºC (75% RH) (Figure 58) than at 40 ºC (75% RH). Again, the degradation was found to be in similar patterns for all microspheres. About 10% of the entrapped drug lost during 90 days of the storage time. Similarly, dP-ENR did not have any influence in drug degradation at 50 ºC. Higher rate of degradation of diclofenac in the microspheres was observed when the microspheres were stored at 70 ºC (75% RH), Figure 59. Similar rate of degradation was observed only the first 20 days of the storage time. Diclofenac in microspheres uncross-link showed faster degradation of diclofenac than that of microspheres cross-link with dP-ENR, which may be due to the acid protons presented at free amino groups of chitosan moiety in uncross-linked microspheres as quaternary ammonium groups were much more than that of cross-linked chitosan in chitosan microspheres cross-linked with dP-ENRs act as a catalyst in diclofenac degradation process.

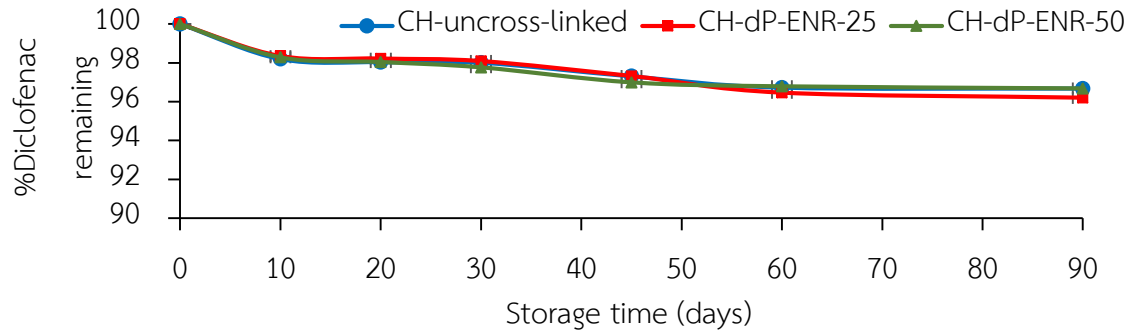


Figure 57. Stability of diclofenac in chitosan microspheres formulations at 40 °C (75% RT).

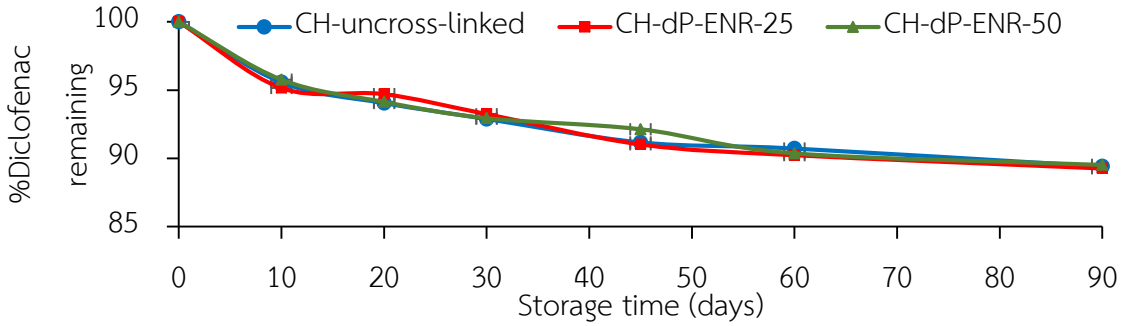


Figure 58. Stability of diclofenac in chitosan microspheres formulations at 50 °C (75% RH).

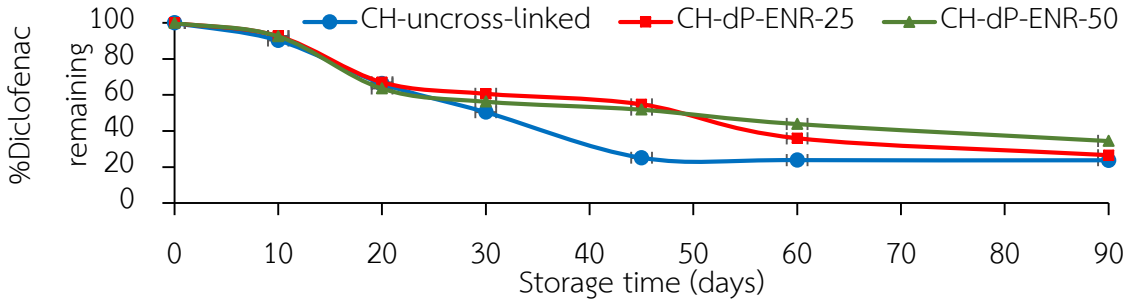


Figure 59. Stability of diclofenac in chitosan microspheres formulations at 70 °C (75% RH).

CH-uncross0linked = Chitosan microspheres uncross-link

CH-dP-ENR-25 = Chitosan microspheres cross-linked with dP-ENR-25

CH-dP-ENR-50 = Chitosan microspheres cross-linked with dP-ENR-50

3.7 Chitosan microspheres in films

The result from previous study indicated that both films and microspheres prepared by chitosan cross-linked with dP-ENR containing diclofenac as a model drug could be applied for skin drug delivery. Since both formulations could up take the drug and release the drug in control pattern, in the next section, we would like to investigate the effect of the combination both films and microspheres to develop film containing microsphere for skin drug delivery.

3.7.1 Optimization for the suitable ratio between chitosan and alginate for chitosan film matrix preparation.

Chitosan films were prepared by dissolving chitosan (CH, 300KDa) mixed with alginate (Al) in 100 mL of 1.0% acetic acid solution. The ratios between CH and Al were varied (CH:Al 1.35g : 0.15g, 1.2g : 0.30g, 1.05g : 0.45g, 0.9g : 0.6g and 0.75g : 0.75g). The solution was stirred to investigate for a suitable ratio for film formulation at room temperature with magnetic stirrer for 24 h. The resulting mixtures were casted on a petri dish and spread manually to the outside borders. The films were dried in an oven at 40°C for 48 h. The suitable ratio was selected on the basis of the clear solution which was obtained.

3.7.2 Chitosan film containing chitosan microspheres cross-linked with dP-ENRs preparation process.

Chitosan films containing chitosan microspheres cross-linked with dP-ENRs were prepared by dissolving chitosan mixed with alginate (Al) (CH: Al, 1.05 : 0.45g) in 100 mL of

1.00% acetic acid solution at room temperature with magnetic stirrer for 24 h. The chitosan microspheres cross-linked with dP-ENR-25 or dP-ENR-50 0.75g were added to the chitosan solution. The resulting mixtures were suddenly casted on a petri dish and spread manually to the outside borders. The films were dried in an oven at 40 °C for 48 h.

3.7.3 Morphology observations

The surface morphologies of chitosan films containing chitosan microspheres cross-linked with dP-ENR-25 or dP-ENR-50 were examined using Scanning Electron Microscopy (SEM, Quanta400, FEI, Czech Republic). Prior to observation, samples were mounted on metal grids, using double-sided adhesive tape, and coated by gold under vacuum before observation.

3.7.4 Determination of drug entrapment efficiency

The chitosan films containing chitosan microspheres cross-linked with dP-ENRs were randomly cut from the whole film and accurately weighed to about 200 mg (n = 3) and were placed in 50 mL methanol in a 100 mL erlenmeyer flask and sonicated for 60 min and left standing at room temperature for 60 min. The clear supernatant was taken to determine the amount of the model drug by using UV-Vis spectrophotometer (Hewlett Packard 8452A, Diode Array Spectrophotometer, USA) ($\boldsymbol{\lambda}$ = 278 nm for diclofenac). The drug entrapment efficiency (%) were calculated using the following equations, (Wang *et al.*, 2009).

$$\text{Drug entrapment efficiency } (\%) = \frac{\text{Actual drug detected}}{\text{Theoretical drug content in each sample}} \text{x} 100$$

3.7.5 *In vitro* release

In vitro release study was performed using modified Franz diffusion cells (Hanson Research Corporation, USA). The diffusion area of the cells was 1.77 cm^3 and the receptor compartment volume was 12 mL. The diffusion cells were connected with a circulating water bath and the temperature was controlled at 37 °C. Phosphate buffer (PBS) pH 5.5 was used as receptor fluid and stirred by externally driven Teflon-coated magnetic bar at 150 rpm. The chitosan film loading chitosan microspheres cross-linked with dP-ENRs containing diclofenac was placed on the receptor compartment, then the donor compartment was connected with a clamp. At suitable time intervals at 0, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 300 and 360 min, an accurate amount (1.0 mL) of each sample solution from the receptor compartment was withdrawn, and replaced with the same volume of PBS at 37 °C. Concentration of model drug in PBS of the receptor compartment was determined by HPLC (Agilent 1100, diode array detector) method. In order to understand the kinetic and mechanism of drug release. Six determinations were carried out for each formulations (n = 6). In the model-dependent approach, various kinetic models were applied to the release profiles, as in Eqs 1 – 5.

First order: $\ln M_t = \ln M_o + K_1 t$ ..... (1)

Higuchi: $M_t = M_o + K_H t^{1/2}$ ..... (2)

Hixson-Crowell: $M_o^3 - M^{t3} = K_{HC} t$ ..... (3)

Baker-Lonsdale: $\frac{3}{2}[1 - (1 - \frac{M_t}{M_\alpha})^{2/3}] \frac{M_t}{M_\alpha} = k_t$ ..... (4)

Korsmeyer-Peppas: $\frac{M_t}{M_\alpha} = K_k t^n$ ..... (5)

where, M_t is the cumulative amount of drug released at any specified time point and M_o is the initial amount of drug in the formulation. K_1, K_H, K_{HC} and K_k are rate constants for first order, Higuchi, HixsonCrowell and Korsmeyer-Peppas models, respectively. In Eq

5, $\frac{\mathbf{M_t}}{\mathbf{M_\alpha}}$ is the percentage of diclofenac released at time t and n is the release exponent that characterizes different release mechanisms. (Dash *et al.*, 2010).

3.7.6 Results and discussion

3.7.6.1 Optimization for a suitable ratio between chitosan and alginate for film matrix preparation.

The suitable wight ratio of chitosan and alginate (Al) needed for film matrix preparation were investigated. The experiments were performed using chitosan mixed with alginate in different ratios (CH:Al, 1.35g:0.15g, 1.2g:0.30g, 1.05g:0.45g, 0.9g:0.6g and 0.75g: 0.75g) to dissolve in 100 mL of 1.0% acetic acid solution (100 mL). A suitable ratio was selected from the final solution after mixing and stirring that gave clear solution without any precipitation or aggregation was observed. The results of the solution after 24 h stirring are summarized in Table 28. The results show that when the ratios of CH:Al were 0.9g:0.6g and 0.75g:0.75g some precipitate were observed in the final solution. High amount of alginate is required in the film formulation, since alginate will provide good elasticity property to the obtained film. Therefore, the ratio of CH:Al at 1.05g:0.45g was selected for the chitosan film matrix preparation. The film matrix will be further used for chitosan film containing microsphere preparation.

3.7.6.2 Morphology observations

The Scanning Electron Micrographs of the chitosan film loaded microspheres cross-linked with dP-ENRs containing diclofenac from different formulations are displayed in Figure 60. SEM micrographs of the surface of chitosan film containing microspheres cross-linked with dP-ENR-25 (Figure 60a) and chitosan film containing microspheres cross-linked with dP-ENR-50 (Figure 60b) showed rough surface with some cracking. The cross-sections of chitosan film containing microspheres cross-linked with dP-ENR-25 and chitosan film containing microspheres cross-linked with dP-ENR-50 showed compact morphology with the microspheres are found distributed throughout the film.

Table 28 Optimization of a suitable ratio of chitosan mixed with alginate for chitosan films preparation.

Amount of chitosan (g)	Amount of Alginate (g)	Characteristic of solutions
1.35	0.15	Clear solution
1.20	0.30	Clear solution
1.05	0.45	Clear solution
0.90	0.60	Solution with precipitate
0.75	0.75	Solution with precipitate

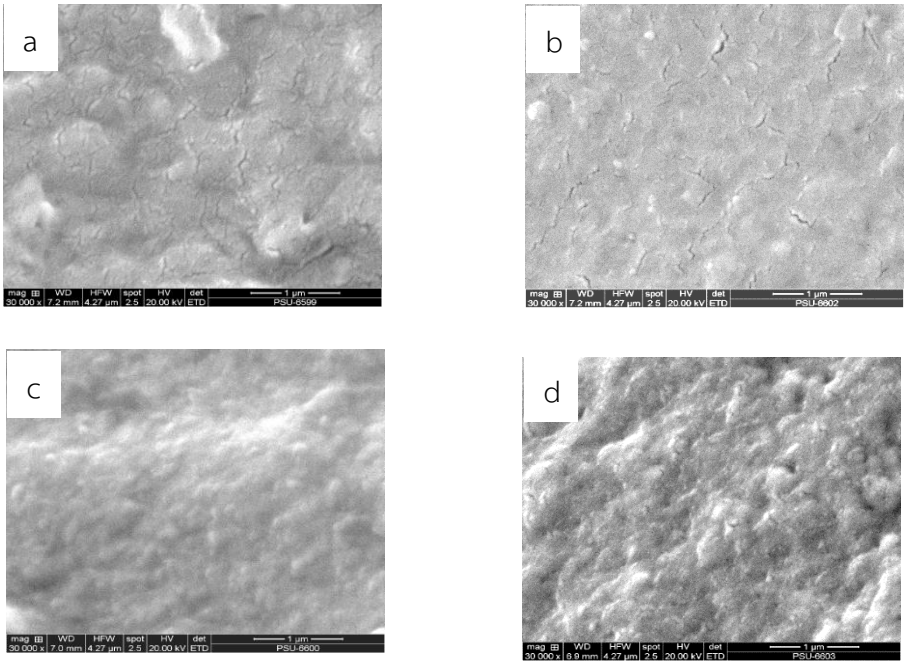


Figure 60. SEM micrographs of chitosan film loaded microspheres cross-linked with dP-ENRs containing diclofenac, (a) surface of CH-dP-ENR-25 microspheres in film, (magnification 30,000x); (b) surface of CH-dP-ENR-50 microspheres in film, (magnification

30,000x); (c) cross-section of CH-dP-ENR-25 microspheres in film, , (magnification 30,000x); (d) cross-section of CH-dP-ENR-50 microspheres in film, (magnification 30,000x).

3.7.6.3 Determination of drug entrapment efficiency

In this study, since diclofenac was previously entrapped in the microspheres cross-linked with dP-ENRs before adding to the solution of chitosan film matrix and casting. The drug entrapment efficiency of the films loaded microspheres containing diclofenac was investigated. The film samples were randomly selected and cut to small pieces, and subjected to the diclofenac content determination by UV-Vis spectroscopy. The drug entrapment efficiencies in films are summarized in Table 29. The amounts of drug entrapped were in a range of 92.59±1.79 and 93.95±0.68%. The results indicated that the microspheres containing diclofenac which were added to the solution of chitosan film matrix were well distributed around the resulting chitosan films which can be observed from small number of SD. values. Moreover, drug lost was observed (about 6-7%) from the preparation process, however, the drugs were still remained in high content.

Table 29 %drug entrapment efficiencies of diclofenac in chitosan films loaded microspheres cross-linked with dP-ENRs containing diclofenac.

Formulations	% drug content in chitosan films (n = 3±SD)
CH-uncross-linked	92.59±1.79
CH-dP-ENR-25	93.95±0.68
CH-dP-ENR-50	93.55±1.31

3.8.6.4 *In vitro* release of diclofenac from the chitosan film loading microsphere containing diclofenac

The *In vitro* release of the drug from chitosan films loading microspheres containing diclofenac was tested using phosphate buffer pH 5.5 and 37°C as a receiving medium. Figure 61 showed that the diclofenac release quickly from uncross-linked chitosan microspheres in films. Diclofenac release from both film containing chitosan microspheres cross-linked with dP-ENRs upto 22% at the first 15 min, and continued releasing to reach 100% at about 4 h. However, sustained release profiles were observed from both formulations of chitosan film containing microspheres cross-linked with dP-ENRs. The slower release form chitosan microspheres in films may be due to the high cross-linked density. Therefore, the drug were penetrated out from the network slowly. The uncross-linked chitosan microspheres in films used in experiment was thin and hydrophilic. It easy for the water to penetrate the film after their contact suggested by (Shi *et al.*, 2009). So, the drug in the film was diffusing quickly and easy dissolving from the film to the water, and release almost 100% drug within 45 min.

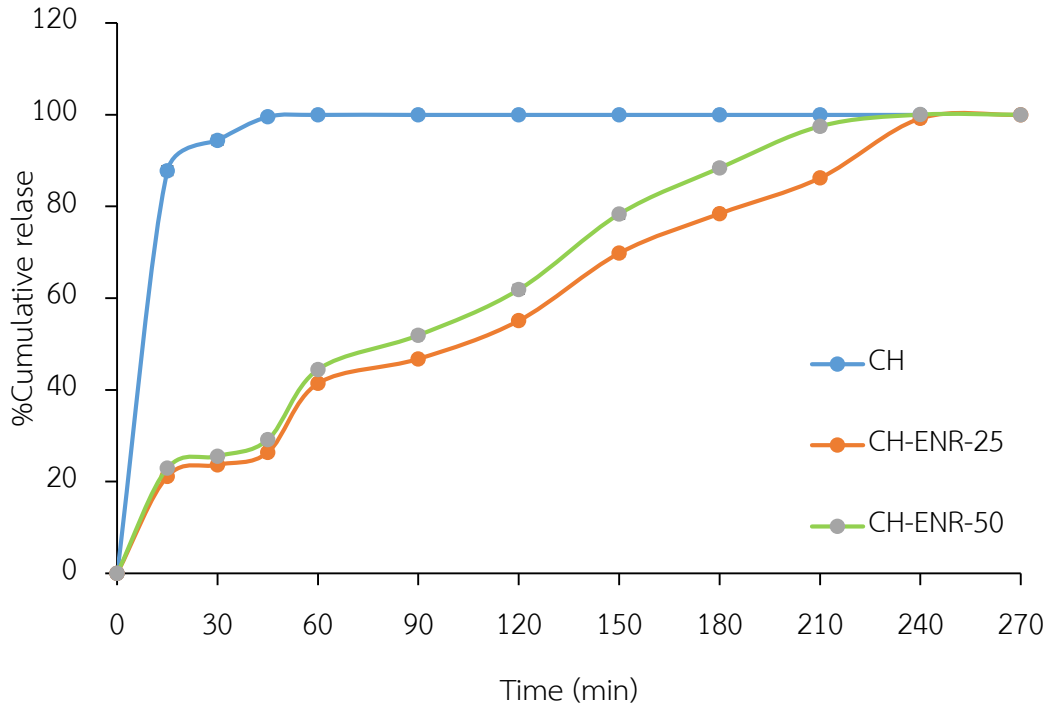


Figure 61. Dissolution profile of diclofenac from chitosan films loading microsphere cross-link with dP-ENRs and uncross-link containing diclofenac (n=6).

3.7.6.5 Release kinetics

The release kinetic patterns were analyzed by using sigma plot fitting program similar to that previously describe. The best fitting model was determined from regression analysis, if the release rate fit with any model it will give the highest regression coefficient. The result indicated that the release kinetic of diclofenac from uncross-linked chitosan microspheres in film were Korsemeyer-peppas model in Fickian diffusion pattern, indicated that the drug was release through the porous network of the chitosan microspheres in films. Similaly, the release kinetic of diclofenac from chitosan microspheres cross-linked with both dP-ENRs were fitted with Korsemeyer-peppas model. However, the first 45 min the drug lease was followed Fickian diffusion pattern but after

45 min the drug release in non Fickain diffusion pattern due to the drug was first diffuse out form microspheres to the film matrix. The drug form the film matrix will be then released to the receiving medium.

Table 30 Kinetics analysis of the release data of diclofenac from chitosan films loaded microspheres cross-linked with dP-ENRs containing diclofenac treated with kinetic equation models (during 0-45 min)*

Film formulation loaded with	First order		Hixon and Crowell		Higuchi model		Baker and Lonsdale		Korsemeyer-peppas model		
	r^2	k	r^2	k	r^2	k	r^2	k	r^2	K	n
CH-dP-ENR-25	0.9679	0.0079	0.9726	0.0022	0.9262	5.4581	0.8485	0.0007	0.9852	2.1474	0.6907
CH-dP-ENR-50	0.9580	0.0092	0.9720	0.0026	0.9099	5.9186	0.8235	0.0008	0.9847	2.0929	0.7191
CH-Uncross-linked	0.7755	0.1348	-0.0201	0.0297	-8.9533	16.9454	0.5863	0.0136	0.9966	64.3629	0.1140

Table 31 Kinetics analysis of the release data of diclofenac from from chitosan films loaded microspheres cross-linked with dP-ENRs containing diclofenac treated with kinetic equation models (during 45-270 min)*

Film formulation loaded with	First order		Hixon and Crowell		Higuchi model		Baker and Lonsdale		Korsemeyer-peppas model		
	r^2	k	r^2	k	r^2	k	r^2	k	r^2	K	n
CH-dP-ENR-25	0.9238	0.0084	0.9645	0.0023	0.9226	5.7736	0.8038	0.0009	0.9851	2.0712	0.6982
CH-dP-ENR-50	0.9258	0.0099	0.9729	0.0027	0.9355	6.1986	0.8153	0.0011	0.9877	3.1870	0.6288

*Sigma plot v.12 was used for the data treatment

CH-dP-ENR-25 = chitosan microsphere cross-linked with dP-ENR-25 containing diclofenac

CH-dP-ENR-50 = chitosan microsphere cross-linked with dP-ENR-50 containing diclofenac

CH-Uncross-linked = chitosan microsphere uncross-link

3.8 CONCLUSIONS

In this study chitosan microspheres were successfully developed by cross-linking reaction of chitosan (MW 300KDa) with dP-ENR-25 or ENR-50. The developed microspheres can be used efficiently to carry bioactive compound, diclofenac. The suitable formulations of chitosan microspheres cross-linked with dP-ENR-25 or ENR-50 containing diclofenac were using 100 mL of 1.5% w/v chitosan in 1% acetic acid solution, 1 mL of 1% dP-ENR-50 or 1 mL of 4% dP-ENR, diclofenac 20 mg and 1 ml of 1% TPP respectively. The resulting solution after mixing was subjected to spray drying to provide microsphere powders.

The obtained microspheres found to have their particle sizes by particle size analyzer in a range of 6-10 μm with low particle size distribution. The results from SEM, however, demonstrated that microparticles were constructed from the agglomeration of a number of nanoparticles. When diclofenac was added to the formulation, light yellow powders were obtained. Their particle sizes by particles size analyzer were in a range of 8.24-10.80 μm. One again by SEM results, the nanospheres were actually obtained, but those nanoparticles were aggregated together or were adsorbed on the surface to produce microspheres. Their zeta potential values of both uncross-linked and cross-linked particles were in a range of +29.74$\pm$2 - +38.42$\pm$3.06 mV, which contributed by the quaternary ammonium group of chitosan. High drug entrapment efficiencies were observed in all formulations imply less drug lost during preparation process. Diclofenac in the microspheres has interaction with the microspheres comparison indicated by FT-IR results. The most important interaction could be contributed by the hydrogen-bonding interaction. Moreover, Diclofenac presented in the microsphere matrix in an amorphous form revealed from the results of DSC and XRD. The results from *in vitro* drug release study demonstrated that microsphere cross-linked with both dP-ENRs can control the drug solubility property in slow release pattern. The release kinetic was fitted with the Korsemeyer-peppas model which the solubility characteristic is depend on the drug

concentration in the matrix. From the stability study found that diclofenac could preserve it stability in the microspheres cross-linked with dP-ENRs than uncross-linked microspheres and the microspheres should be stored at temperature lower than 40 °C in controlled humidity condition.

The resulting microspheres cross-linked with dP-ENRs containing diclofenac were further utilized (0.75g) as additive in chitosan film matrix. The chitosan film matrix was prepared by using CH:Al in a range 1.05g : 0.45g ratio to dissolve in 100 mL 1% acetic acid solution. The chitosan films were obtained by casting method. The SEM revealed that the added microspheres were well distributed throughout the film matrix. No drug lost was observed from the preparation process resulting in high drug entrapment efficiency. From the drug release profile determination suggested that the drug from microspheres would be first dissolved to the chitosan matrix and then diffuse out to the medium resulting in slow release pattern. The release was in accordance with the Korsemeyer-peppas model. Faster release was observed in the film loading with uncross-linked chitosan microsphere.

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CONCLUSION

From all of our investigations, the results demonstrated that chitosan films and chitosan microspheres can be prepared by using epoxidized natural rubber (ENR) as a cross-linking agent. In order to use the developed system for skin drug delivery application, ENR-25 and ENR-50 were subjected to remove any proteins remaining before using for the investigation to provide deproteinated ENRs.

The suitable formulations for preparation of chitosan film cross-linked with deproteinated ENR (dP-ENR) containing diclofenac as a model drug were using 100 mL of 1.5% w/v of chitosan (MW 300KDa) mixed with 1 mL of 1% w/v of dP-ENR-25 or 4% of dP-ENR-50 in THF while stirring, diclofenac (20 mg) was previously dissolved in 5 mL methanol and added dropwise to the chitosan solution and continue stirrings for 24 h at RT. The resulting solutions were then casted on a petri dish and dry at 40 °C to give dried films. The improved properties including film swelling, moisture uptake capacity as well as mechanical properties (tensile strength and elongation at break) were observed in the obtained chitosan films compared to that the film without cross-linking reaction. The cross-linking reaction would occur via nucleophilic ring opening reaction in acetic medium to form 3 dimension networks that contributed to the improved properties. Moreover, the developed films showed no skin irritation, therefore, they are suitable for skin drug delivery. Diclofenac loaded chitosan films cross-linked with dP-ENRs were found to have high drug entrapment efficiencies (95-96%) indicating that less drug lost during preparation process. The drug were well distributed across the film without any precipitation was observed. Diclofenac was in an amorphous formed indicated by DSC and XRD results. Sustained release pattern according to the Korsemeyer-peppas model could be due to the drug interaction with chitosan moiety in film formulation. The results from the stability study revealed that the drugs could

preserve their stability when the films are stored at low temperature in a controlled humidity.

Chitosan microspheres were successfully developed by using chitosan cross-linked with dP-ENRs. In the microsphere preparation formulation, 100 mL of 1.5% w/v chitosan (MW 300KDa) in 1% acetic acid was mixed with 1 mL of 1% w/v of dP-ENR-50 or 4% w/v of dP-ENR-25, diclofenac (20 mg) was previously dissolved in 5 mL methanol and added dropwise to the chitosan solution, 1 mL of 1% aqueous solution of TPP was then added and continued stirring to get clear solution. The resulting solution was subjected to the spray drying process. Spray-drying conditions such as nozzle (500 μm), inlet temperature, liquid flow, and compressed spray air flow (represented as the volume of the drying air input) were set at 175°C, 2 mL/min, 1.2 m^3/min, respectively. The product were obtained as light yellow powders having their particle size in a range of 10.35-10.89 μm (PI = 0.82-0.92) analyzed by particle size analyzer with positive zeta potential values. However, SEM micrographs indicated that the microparticles were constructed from the aggregation of the nanoparticles. The nanoparticles were in spherical shapes having sizes less than 1 μm. Diclofenac in the chitosan microspheres cross-linked with dP-ENRs was well entrapped (～94% entrapment efficiency) and dispersed in the microspheres matrix in amorphous form. The drug could release out from the microspheres slower than the uncross-linked microspheres. Most of the entrapped drug could be released within 3 h in slow released pattern following Hixon and Crowell model. Therefore, the dissolution of the drug was due to the charge in the microspheres' and the large surface area to promote drug diffusion.

The developed chitosan microspheres cross-linked with dP-ENRs were further applied to chitosan-alginate blended films. The chitosan-alginate blended film matrix was prepared by mixing chitosan (1.05 g) with alginate (0.45 g) in 100 mL of 1% acetic acid solution. The developed microspheres cross-linked with dP-ENRs containing diclofenac drug were then added to the mixtures and continued casting at 40 °C in an oven to give chitosan films loading microspheres cross-linked with dP-ENRs containing diclofenac. The obtained films showed smooth surface with little cracking. Film's cross section indicated that the adding microspheres were well distributed across the film

matrix. High drug entrapment efficiency was observed and the drug was disperse uniformity. Diclofenac was found much slower released out from the films than the films containing uncross-linked microspheres. The release profile of diclofenac displayed sustained release pattern in which following Korsemeyer-peppas model for the first 45 min and Korsemeyer-peppas model from 45-270 min. The results indicated that the drug could be distributed out from the microspheres to the film matrix and the drug will be then dissolved out from the matrix to the medium.

The results demonstrated the potential utilization of epoxidized natural rubber as an economical cross-linking agent for the preparation of films and microspheres for drug delivery application. However, suitable formulations should be selected for the preparation process to provide the desired properties of the products.

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APPENDIX

METHOD VALIDATION

1. INTRODUCTION

Diclofenac is 2-(2-(2,6-dichlorophenyl amino) phenyl) acetic acid (Table 2). It is a non-steroidal anti-inflammatory drug (NSAID) with anti-inflammatory, antipyretic, and analgesic action as a result of its ability to block prostaglandin synthesis by inhibition of the cyclooxygenase (COX) enzyme. (Basavaraj *et al.*, 2008, Bv *et al.*, 2008, Chowdhury *et al.*, 2011, Basarkar *et al.*, 2013). Many publications have described the use of bio-materials to produce effective anti-inflammatory formulations of this therapeutic agent, e.g. in beads or microspheres (Fernández-Hervás *et al.*, 1998, Çalış *et al.*, 2002, González-Rodríguez *et al.*, 2002, Basavaraj *et al.*, 2008, Bv *et al.*, 2008, Al-Kahtani Ahmed *et al.*, 2009, Basarkar *et al.*, 2013, Al-Kahtani and Sherigara, 2014, Cooper and Harirforoosh, 2014) and films (Singh *et al.*, 1993, Kramar *et al.*, 2003, Ahmed *et al.*, 2009, Jadhav *et al.*, 2009, Liu *et al.*, 2010, El-Sousi *et al.*, 2013). Several analytical methods have been reported in the literature for the determination of diclofenac in pharmaceutical and biological fluids, including spectrophotometric (Mehta *et al.*, 2008, Ioele *et al.*, 2014, Pandya *et al.*, 2014) and chromatographic (Davarani *et al.*, 2012, Song *et al.*, 2012) methods. High Performance Liquid Chromatography (HPLC) normally provides more accurate determination of the drug than spectrophotometric methods especially in the presence of interfering matrices that result from the different formulations. However, most of the available HPLC methods may not be the best analytical methods of choice when it is required to increase the throughput and reduce analysis costs compared to the UV visible spectrophotometric methods. For routine quality control work, hundreds of samples need to be analyzed on a daily basis and HPLC methods are very costly in terms of

time and resources. A UV visible spectroscopic (UV-vis) method is known to be a simpler, faster and more economical method for the detection and quantification compared to HPLC. Both HPLC and UV-vis methods have been employed for the determination of diclofenac in microspheres and film formulations (Singh *et al.*, 1993, De Souza and Tubino, 2005, Liu *et al.*, 2010, Narayana Charyulu *et al.*, 2014, Oliveira *et al.*, 2014). However, no literature has reported a validated method for the quantitative determination of diclofenac in films and microsphere formulations. Method validation is a process of establishing that the performance characteristics of the analytical method are suitable for the intended application. The validation method process for analytical procedures begins with the planned and systemic collection by the applicant of the validation data to support analytical procedures(Ravichandran *et al.*, 2010). Moreover, the analytical methods of pharmaceutical products should be validated according to the International Conference on Harmonization (ICH) guidelines for validation of analytical procedures (I. C. H. Harmonized Tripartite Guideline, 1997). This part of the work is therefore aimed to validate and compare the effectiveness between two analytical procedures, UV-vis and HPLC methods for the determination of diclofenac in chitosan films and chitosan microspheres.

2. Chemicals and reagents

- Diclofenac raw material was obtained from China.
- Methanol (AR grade) was purchased from Labscan Ltd., Bangkok, Thailand.
- Sodium dihydrogen orthophosphate (AR grade) was received from Finechem Pty Ltd, Australia.

3. Instrumentation

The HPLC analyses were carried out on an Agilent 1100, and diode array detector with Chemstation software. The chromatographic system including a reversed phase ACE® C8, 5 µm, 4.6 x 150 mm HPLC column (ACE, Scotland) as the stationary phase, a mixture of methanol: phosphate buffer pH = 2.5 (66: 34 by volume) was the mobile phase at a flow rate of 1.0 mL/min. Detection of diclofenac was performed by measuring the absorption at 254 nm and the injection volume was 20 µg/mL.

The UV-vis spectrophotometer was a Hewlett Packard 8452A, Diode Array Spectrophotometer, USA with a 1 cm path-length, a quartz cuvette was used for the measurement of the absorption of diclofenac at 278 nm. The spectral bandwidth was set at 2 nm. All weights were measured on an analytical balance (Sartorious® model BSA 2248)

4. Preparation of standard solutions

A stock solution of diclofenac was prepared by dissolving 100 mg of the diclofenac reference standard in methanol in a 100 mL volumetric flask to give a 1 mg/mL solution of diclofenac. This solution was stored at 4 °C until used. The stock solution was then serially diluted with the mobile phase to provide calibration standard solutions of 5, 10, 15, 20 and 25 µg/mL. These standard solutions were used for both the HPLC and UV-vis methods.

5 Sample Preparation

The chitosan films or chitosan microspheres (100 mg) were placed in 50 mL of methanol in a 100 mL erlenmeyer flask and sonicated for 60 min then left standing at room temperature for 60 min. The clear supernatant was taken and filtered through a filtering membrane (0.45 µm) before being used for the determination process by HPLC and UV visible spectrophotometry.

6 Validation of the assay

1.6 UV-Visible spectrophotometric

6.1.1 Linearity and calibration curve

Diclofenac standard solutions of 5, 10, 15, 20 and 25 µg/mL were prepared in methanol form the diclofenac stock standard solutions (100 µg/mL). Three replicate analyses of each solution were performed in a day. Three determinations were carried out for each solution (n = 3). Linearity was obtained by plotting the absorbance value against the concentrations of the standard solutions and analyzed using the linear least squares regression equation. Linearity was expressed as a coefficient of determination (r^2) which should be more than 0.999.

6.1.2 Precision

The measurements of intra-day and inter-day variability were utilized to determine the precision of the method. For intra-day precision, five concentration levels of diclofenac standard solutions at 5, 10, 15, 20 and 25 µg/mL in the calibration range were prepared with methanol in triplicate (n = 3). For inter-day precision of diclofenac standard solutions was assessed by repeating the intra-day precision on three different days. Concentrations of diclofenac standard solutions from the

experiments were calculated with a linear equation of the standard curve. Precision was calculated and expressed as percentage relative standard deviation (%RSD).

6.1.3 Accuracy

The accuracy of the method was performed by recovery study by using the method of standard additions. The recovery was determined by recovery of known amounts of diclofenac reference standard used at three concentration levels of 1.84, 2.30 and 2.76 µg/mL. The blank microspheres (300 mg) or blank films (1 inch x 1 inch) were placed in 50 mL of methanol in a 100 mL erlenmeyer flask and added diclofenac reference standard. The samples were sonicated for 45 min and left standing at room temperature for 60 min. All solutions were prepared in triplicate. The clear supernatant was taken to determine the amount of diclofenac by using UV spectrophotometer and calculating to percentage recovery. The percentage recovery of diclofenac was calculated with a linear equation of the standard curve.

6.1.4 Specificity

The specificity is a method producing a response for only a single analyst accurately and specifically in the present of components in the sample matrix. The specificity of the analytical method was confirmed by analysis of 10 µg/mL of diclofenac standard solution. The UV-Visible spectrum of diclofenac was recorded in a range of 190-390 nm as follow previously mentioned. The identification was performed by assessing the presence of only diclofenac spectrum at 278 nm without other spectrum. The blank chitosan film cross-link with ENR were prepared by dissolving chitosan 1.5 g (MW = 300,000) in 1% acetic acid solution (100 mL) at room temperature (30$\pm$1 ^{o}C) with magnetic stirrer for 24 h. Solution of ENR-25 or ENR-50 in tetrahydrofuran was added to the chitosan solution and stirred at room temperature (30$\pm$1 ^{o}C) for 24 h. The resulting mixtures were casted on a petri dish and spread manually to the outside borders. The films were dried in an oven at 40^{o}C for 24 h. The blank chitosan microspheres cross-link with ENR were prepared by dissolving chitosan 1.50 g (MW = 300,000) in 1% acetic acid solution (100 mL) at room temperature (30$\pm$1 ^{o}C) with magnetic stirrer for 24 h. Solution of ENR-25 or ENR-50 in tetrahydrofuran (1% w/v, 1

mL) was added to the chitosan solution and stirred at room temperature (30±1 °C) for 24 h. Aqueous solution of tripolyphosphate was added dropwise to the solution and stirred at room temperature (30±1 °C) for 24 h. The resulting mixture was then spray dried. Spray-drying conditions such as nozzle (500 µm), inlet temperature, liquid flow, and compressed spray air flow (represented as the volume of the drying air input) were set at 175 °C, 2 mL/min, 1.2 m3/min, respectively.

6.1.5 Limit of detection (LOD) and limit of quantitation (LOQ)

Limit of detection is the lowest concentration of analyst that is detectable at the most sensitive instrument settings, but not necessarily quantitated, under the stated experimental conditions. Limit of quantification is the lowest concentration of analyst that can be determined with acceptable precision and accuracy, under the stated experimental conditions. Standard solutions of diclofenac were analyzed in the range of 5, 10, 15, 20 and 25 µg/mL. LOD and LOQ were determined on the basis of response and slope of the regression equation from the calibration curve using the following equations.

$$LOD = 3.3\sigma/S$$

$$LOQ = 10\sigma/S$$

6.2 HPLC

6.2.1 Linearity and range

Linearity is the ability of the method to produce test results that are proportional, either directly or by a well-defined mathematical transformation, to the concentration of analyst in samples within a given range. Range is the interval between the upper and lower levels of analyst (including these levels) that have been demonstrated to be determined with the required precision, accuracy and linearity. Diclofenac standard solutions of 2, 5, 10 and 15 µg/mL were injected into the HPLC

system. Three replicate analyses of each solution were performed in a day (3 injections × 4 concentrations × 3 replicates). Three determinations were carried out for each solution (n = 3). Linearity was obtained by plotting the peak areas against concentrations of the standard solutions and analyzed using the linear least-squares regression equation. Linearity was expressed as a coefficient of the determination (r^2) which should be more than 0.999.

6.2.2 Precision

The measurements of intra-day and inter-day variability were utilized to determine the precision of the method. For intra-day precision, four concentration levels of diclofenac standard solutions at 2, 5, 10 and 15 µg/mL in the calibration range were prepared with methanol in triplicate (n = 3). For inter-day precision of diclofenac standard solutions was assessed by repeating the intra-day precision on three different days. Concentrations of diclofenac standard solutions from the experiments were calculated with a linear equation of the standard curve. Precision was calculated and expressed as percentage relative standard deviation (%RSD).

6.2.3 Accuracy

The accuracy of the method was performed by recovery study by using the method of standard additions. The recovery was determined by recovery of known amounts of diclofenac reference standard used at three concentration levels of 1.84 (80%), 2.30 (100%) and 2.76 (120%) µg/mL. The blank films size is 1" x 1" in a square and blank microspheres (100 mg) were separately placed in 50 mL methanol in a 100 mL erlenmeyer flask. Each diclofenac concentration was added in the blank film and blank microspheres solutions and sonicated for 90 min and left standing at room temperature for 60 min. The clear supernatant was taken to determine the amount of the diclofenac standard by using HPLC. All solutions were prepared in triplicate and assayed (3 injections × 3 concentrations × 3 replicates). The percentage recovery of diclofenac standard was calculated with a linear equation of the standard curve.

6.2.4 Limit of detection (LOD) and limit of quantitation (LOQ)

Limit of detections is the lowest concentration of analyst that is detectable at the most sensitive instrument settings, but not necessarily quantitated, under the stated experimental conditions. Limit of quantification is the lowest concentration of analyst that can be determined with acceptable precision and accuracy, under the stated experimental conditions. Standard solutions of diclofenac were analyzed in the range of 2, 5, 10 and 15 µg/mL. LOD and LOQ were determined on the basis of response and slope of the regression equation from the calibration curve. Using the following equations.

$$LOD = 3.3\sigma/S$$

$$LOQ = 10\sigma/S$$

7 Statistical analysis

Results are expressed as the mean$\pm$S.D. of at least three experiments. Analysis of variance (ANOVA) was used to test the statistical significance of differences among groups. The SPSS software package (SPSS 17.0 for Windows, SPSS Inc., Chicago, IL, USA). Comparison of means was performed by Duncan's test with confidence level as $P < 0.05$.

8 Result and discussion

8.1 Validation of the UV-Visible spectrophotometric method

Method validation is a process of establishing that the performance characteristics of the analytical method are suitable for the intended application. The methods validation process for analytical procedures begins with the planned and systemic collection by the applicant of the validation data to support analytical procedures (FDA, 2000). The methods were validated according to International Conference on Harmonization (ICH) guidelines for validation of analytical procedures (ICH, 1996). The aim of this work was developed and validated of UV methods for the estimation of diclofenac in chitosan films and chitosan microspheres. The UV-Visible spectrum recorded at 278 nm, which is a maximum absorption wavelength of diclofenac. The results from the method validation are as followed:

8.1.1 Linearity

The calibration curve for standard diclofenac solutions was obtained by plotting the absorbance values versus concentrations of standard solutions of diclofenac in methanol. Linearity was found to be in the range of 5-25 µg/mL (Figure 62) with significantly high value of correlation coefficient r^2 = 0.9996; the representative equation was y = 0.0445x + 0.006. The quantitative parameters for determination of diclofenac are listed in Table 32. The low R.S.D value (<2%) indicated the high precision of the calibration curve and accepted for further analysis method. It should be noted that at a time of the determination, a new standard curve is constructed for each experiment interpretation.

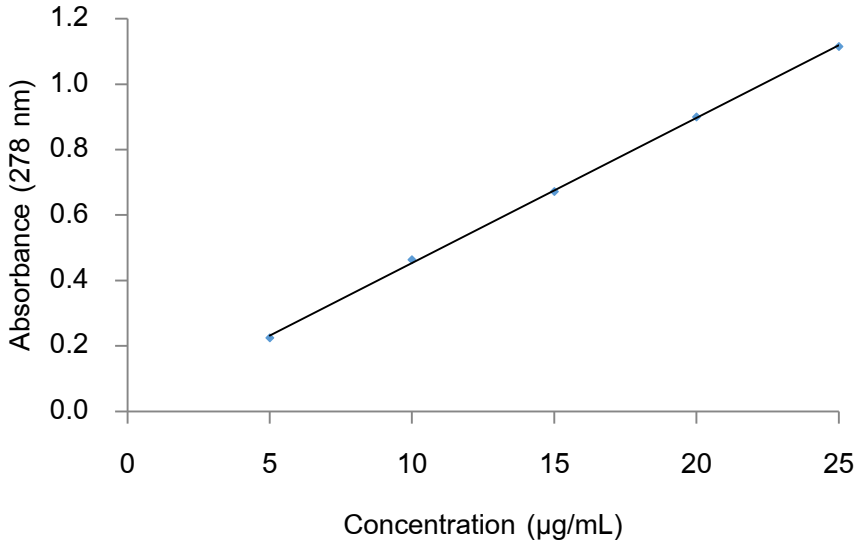


Figure 62. Calibration curve of diclofenac standard solutions (5-25 µg/mL) in methanol recorded at 278 nm.

Table 32 Quantitative parameters for diclofenac determination by UV-Vis spectrophotometry (solvent = methanol).

Parameters	**Regression analysis results**
λ max (nm)	278
Regression coefficient	0.9996
Slope ± standard deviation	0.0445 ± 0.0001
Intercept ± standard deviation	0.006 ± 0.0046
Relative standard deviation (%)	0.67
Concentration range (µg/mL)	5.0 - 25.0
Number of points	5

8.1.2 Precision

The precision analyses result of intra-day (n = 3) and inter-day (n = 3) of various concentrations of diclofenac solutions were obtained and reported as %R.S.D. values. The values of intra-day and inter-day precision were 0.02 – 1.37% (Table 33) and 0.23 – 0.55% (Table 34), respectively which lower than 2% indicated that the developed method gave a trust precision value. It is therefore, the method was suitable for using in determination the entrapping efficiencies of diclofenac in films or microspheres formulations (Figures 63 and 64).

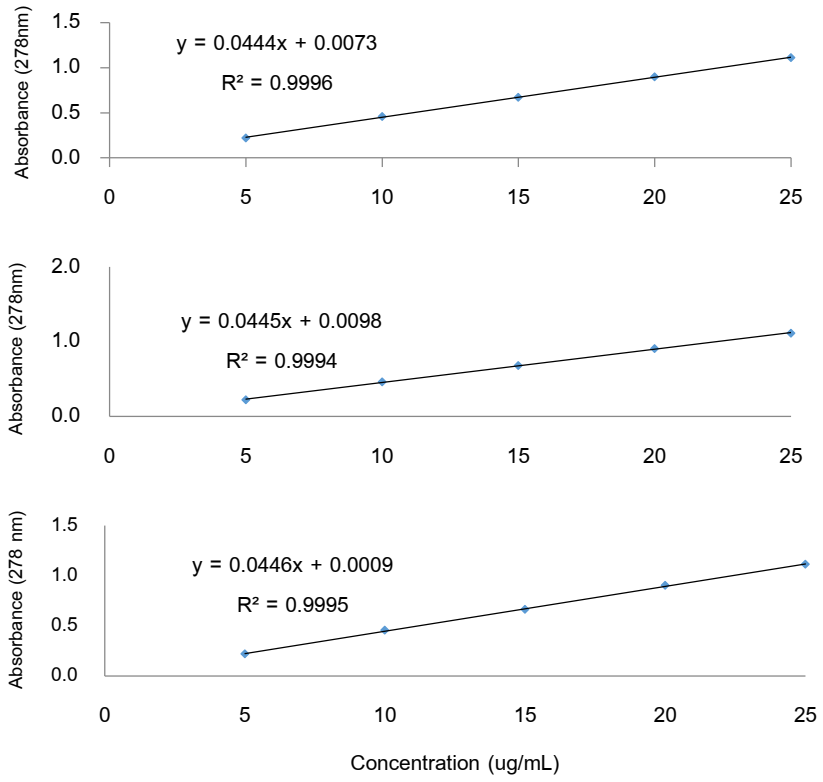


Figure 63. Intra-day precision of five different concentrations of diclofenac solutions (n = 3); (average R.S.D. = 0.40%)

Table 33 Intra-day precision of diclofenac solutions of 5, 10, 15, 20 and 25 µg/mL.

Conc. of diclofenac (µg/mL)	mean ± SD (µg/mL) of absorbance values at 278 nm (n = 3)	% R.S.D.	Average % R.S.D.
5	0.22±0.0016	0.38	
10	0.46±0.0370	0.23	
15	0.67±0.0092	0.29	0.40
20	0.90±0.0041	0.53	
25	1.11±0.0002	0.55	

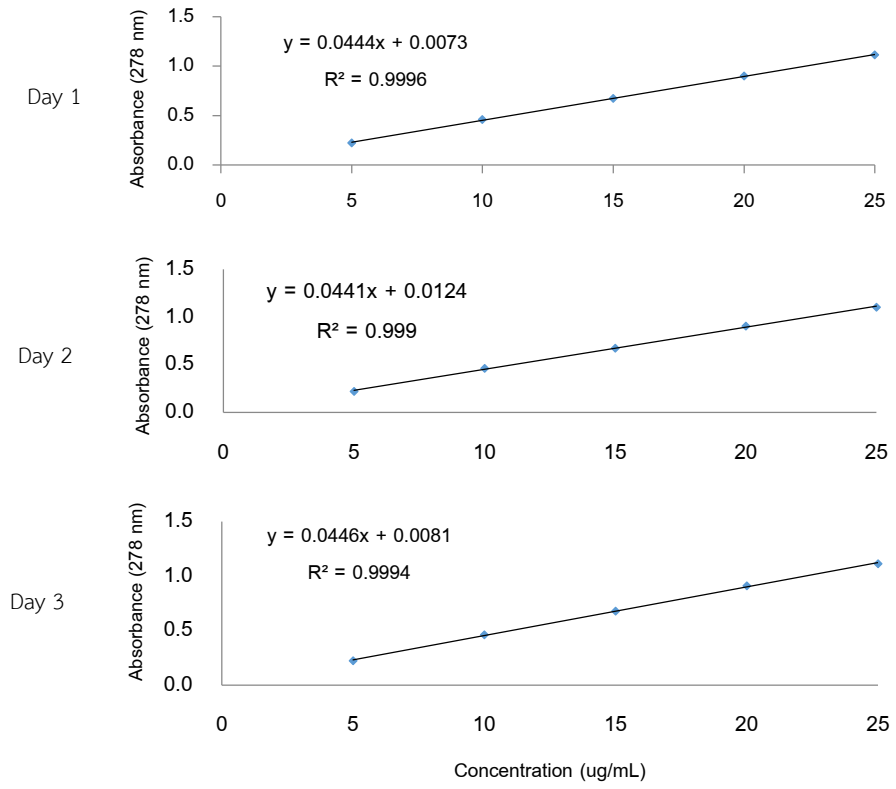


Figure 64. Inter-day precision of five different concentrations of diclofenac solutions (n = 3); (average R.S.D. = 0.77 %).

Table 34 Inter-day precision of diclofenac solutions of 5, 10, 15, 20 and 25 µg/mL.

Conc. of diclofenac (µg/mL)	mean ± SD (µg/mL) of absorbance values at 278 nm (n = 3)	% R.S.D.	Average % R.S.D.
5	0.21± 0.0017	0.82	
10	0.44± 0.0037	0.85	
15	0.65±0.0046	0.70	0.77
20	0.88±0.0065	0.74	
25	1.07±0.0002	0.75	

8.1.3 Accuracy

Accuracy was investigated by means of a standard diclofenac addition experiment at three concentration levels (1.84, 2.30 and 2.76 µg/mL) in triplicate. The mean recoveries of each concentration are shown in Tables 35-36, which assured the method accuracy.

Table 35 Recovery (%) of diclofenac solutions of 1.84, 2.30 and 2.76 µg/mL. (Films)

Concentration of added diclofenac (µg/mL)	Amount of diclofenac found (µg/mL)			Mean ± SD (µg/mL)	Recovery (%) (Mean±SD)
	I	II	III		
1.84	1.90	1.82	1.85	1.85 ± 0.04	101.01 ± 2.03
2.30	2.29	2.29	2.24	2.27 ± 0.03	98.88 ± 1.36
2.76	2.78	2.71	2.75	2.75 ±0.04	99.50 ± 1.36

Table 36 Recovery (%) of diclofenac solutions of 1.84, 2.30 and 2.76 µg/mL.

Concentration of added diclofenac (µg/mL)	Amount of diclofenac found (µg/mL)			Mean ± SD (µg/mL)	Recovery (%) (Mean±SD)
	I	II	III		
1.84	1.81	1.86	1.81	1.83 ±0.03	99.38 ± 1.56
2.30	2.32	2.31	2.28	2.31 ± 0.02	100.22 ± 0.88
2.76	2.79	2.78	2.74	2.77 ± 0.03	100.44 ± 1.11

8.1.4 Specificity

No other absorption band was found interference at 278 nm in the UV-Visible spectra of sample solution prepared from blank films. Only absorbance peak of diclofenac was found in the UV-Visible spectra of sample solutions prepared from films or microspheres containing diclofenac (Figure 65). Therefore, the determination of diclofenac was performed at 278 nm to evaluate the presence of absorption band of sample solution at 278 nm and indicative of diclofenac in formations.

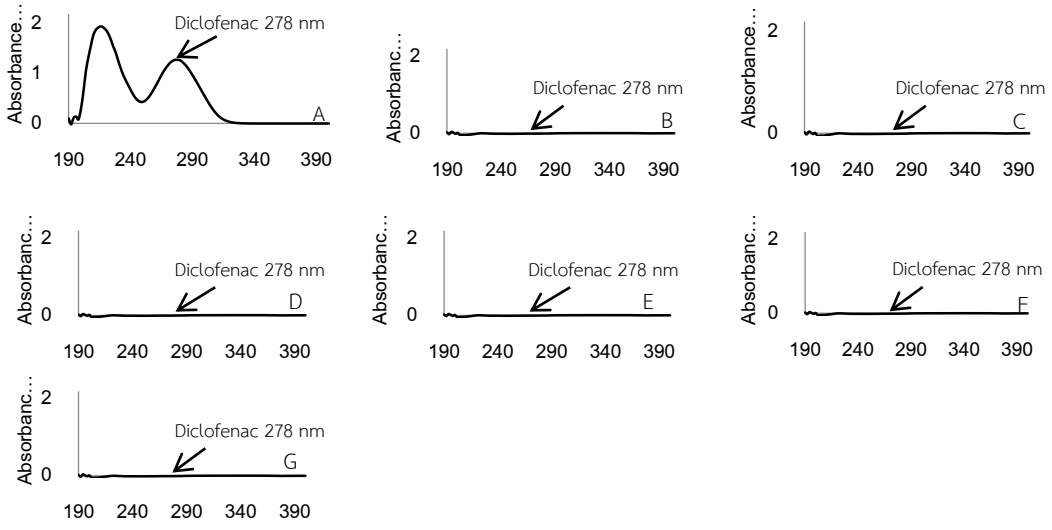


Figure 65. Specificity of the method displayed as UV-vis spectra of diclofenac (A), blank chitosan film (B), blank chitosan film cross-linked with dP-ENR-25 (C), blank chitosan film cross-linked with dP-ENR-50 (D), blank chitosan microspheres (E), blank chitosan microspheres cross-linked with dP-ENR-25 (F), blank chitosan microspheres cross-linked with dP-ENR-50 (G).

8.1.5 Detection and quantification limit

Detection and quantification limits in the UV-Visible spectrophotometric analysis was optimized using diluted solutions of diclofenac in a range at 5.0-25.0 µg/mL. The limit of detection (LOD) was calculated using parameters of the calibration curve and gave 0.12 µg/mL. The limit of quantification (LOQ) was determined based on the parameters of the calibration curve and gave 0.36 µg/mL.

8.2 Validation of the chromatographic method (HPLC) for quantitative analysis

8.2.1 Linearity and range

The solutions of diclofenac were prepared in a range of 2-15 µg/mL and utilized in the linearity testing. The analytical method was performed as previously described in section 2.3.1 the peak areas obtained from each run were plotted against the concentrations. Each concentration was prepared in triplicate and relative standard deviation (%) was determined. The linear fitted equation (Y = aX + b) and correlation coefficient (r^2) were summarized in Table 37. The result demonstrated that diclofenac solutions displayed linear response correlated to concentration range (Figure 66).

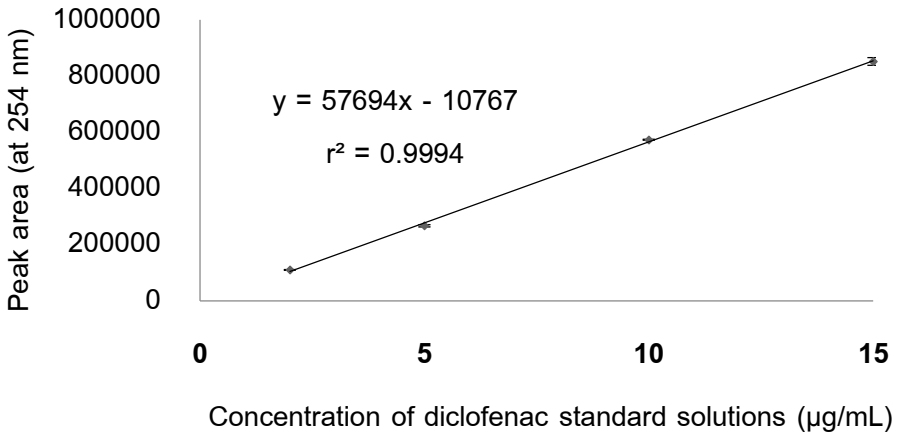


Figure 66. Calibration curve of diclofenac standard solutions (mean ± SD; n = 3) determination by the HPLC method.

Table 37 Quantitative parameters for diclofenac determination by HPLC method (solvent = methanol).

Parameters	Regression analysis results
λ max (nm)	254
Regression coefficient	0.9994
Slope $\pm$ standard deviation	57694 $\pm$ 849
Intercept $\pm$ standard deviation	10767 $\pm$ 4095
Relative standard deviation (%)	1.10
Concentration range (µg/mL)	2.0 - 15.0
Number of points	4

8.3.2 Precision

The precision analyses result of intra-day (n = 3) and inter-day (n = 3) of various concentrations of diclofenac standard solutions were obtained and reported as %R.S.D. values. The values of intra-day and inter-day precision were 0.34 – 1.62% (Table 38) and 1.85 – 2.66% (Table 39), respectively which lower than 5%. The values indicated that the developed method gave a trust precision value. It is therefore, the method was suitable for using in determination the entrapping efficiencies of diclofenac in films or microspheres formulations (Figures 67 and 68).

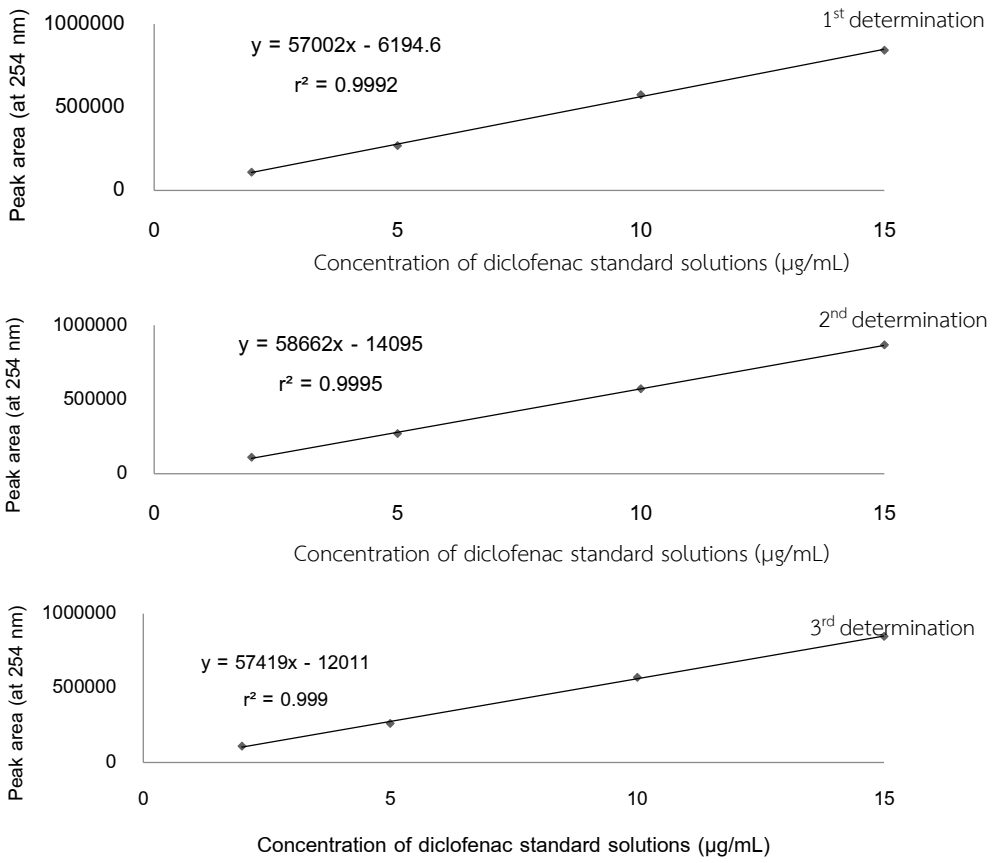


Figure 67. Intra-day precision of four different concentrations of diclofenac standard solutions (n = 3); (average R.S.D. = 1.10%).

Table 38 Intra-day precision of diclofenac standard solutions of 2, 5, 10 and 15 µg/mL.

Conc. of diclofenac standard solutions (µg/mL)	mean ± SD (µg/mL) of peak areas measured at 254 nm (n = 3)	% R.S.D.	Average % R.S.D.
2	109975±944	0.86	1.10
5	267085±4317	1.62	
10	573496±1968	0.34	
15	852594±13526	1.59	

* R.S.D. (%) = (SD/mean) × 100; [a] All values were obtained from triplicate analyses (n=3).

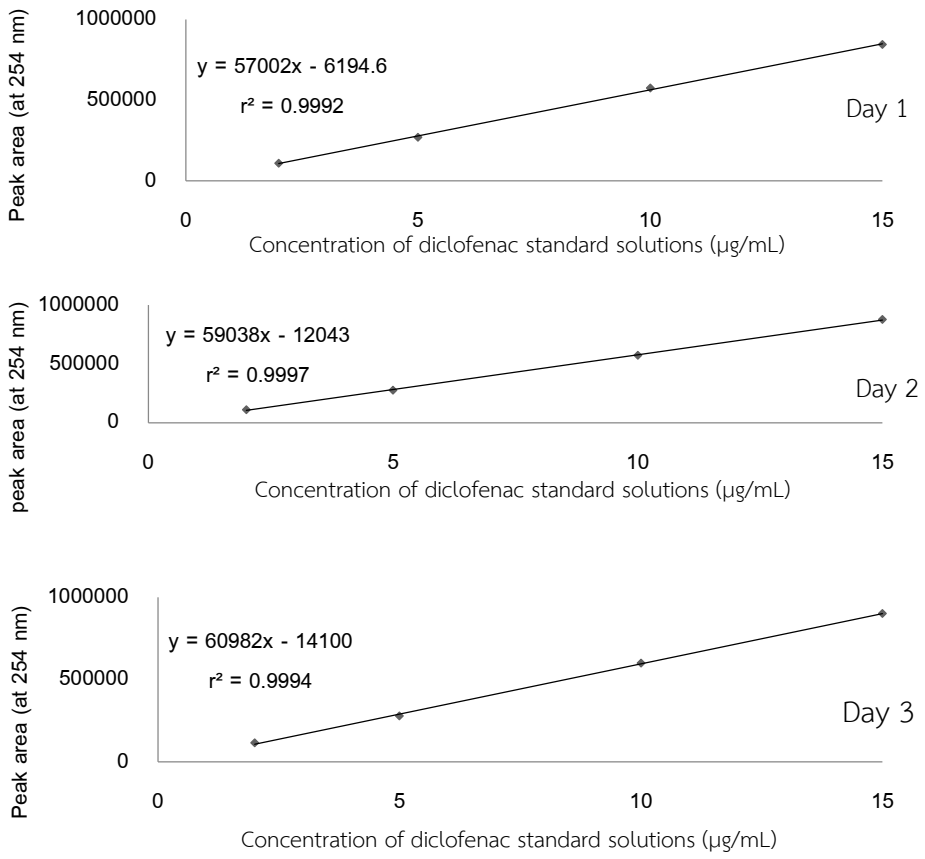


Figure 68 Inter-day precision of four different concentrations of diclofenac solutions (n = 3); (average R.S.D. = 2.18 %).

Table 39 Inter-day precision of diclofenac standard solutions of 2, 5, 10 and 15 µg/mL.

Conc. of diclofenac standard solutions (µg/mL)	mean ± SD (µg/mL) of peak areas measured at 254 nm (n = 3)	% R.S.D.	Average % R.S.D.
2	112747±2594	2.30	2.18
5	275528±5104	1.85	
10	581626±15524	2.66	
15	882398±16889	1.91	

* R.S.D. (%) = (SD/mean) × 100; [a] All values were obtained from triplicate analyses (n=3).

8.2.3 Accuracy and precision

The recovery of the method was performed by recovery study using standard addition method. Standard solutions of diclofenac at three different concentration levels (1.84, 2.30 and 2.76 µg/mL), were added with blank film or microsphere. All samples were prepared in triplicate and the percentage recovery were determined. The recovery (%) results of each concentration are shown in table 40-41. The percent recovery of the method was found to be in a range of 92.44 – 99.52 % .It demonstrated that the analytical method showed good accuracy.

Table 40 Recovery (%) of diclofenac solutions of 1.84, 2.30 and 2.76 µg/mL. (Films).

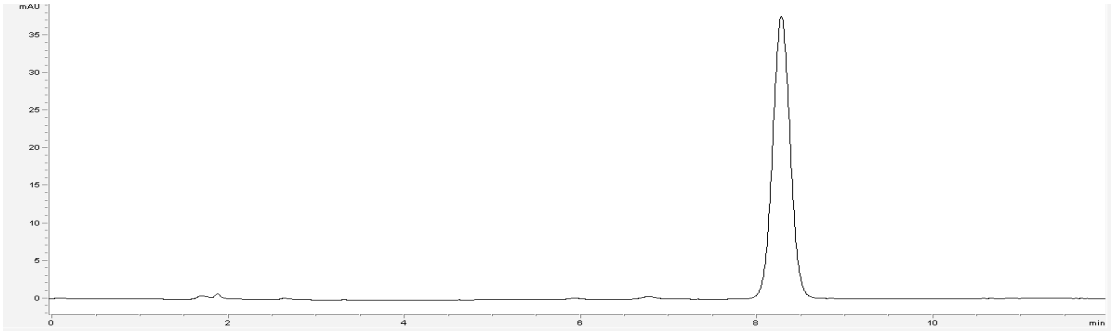
Concentration of added diclofenac (µg/mL)	Amount of diclofenac found (µg/mL)			Mean ± SD (µg/mL)	Recovery (%) (Mean±SD)
	I	II	III		
1.84	1.97	1.77	1.76	1.83 ± 0.12	99.52 ± 6.38
2.30	2.15	2.22	2.21	2.19 ± 0.04	95.40 ± 1.56
2.76	2.60	2.60	2.52	2.57 ± 0.04	93.17 ± 1.49

Table 41 Recovery (%) of diclofenac solutions of 1.84, 2.30 and 2.76 µg/mL. (Microspheres).

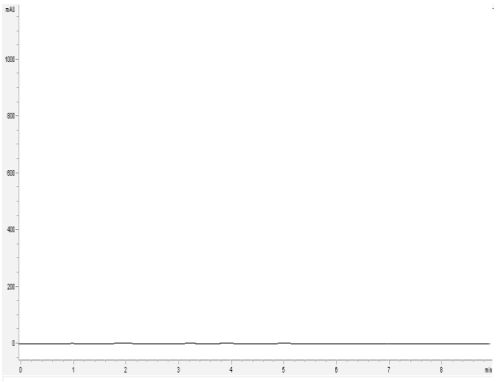
Concentration of added diclofenac (µg/mL)	Amount of diclofenac found (µg/mL)			Mean ± SD (µg/mL)	Recovery (%) (Mean±SD)
	I	II	III		
1.84	1.78	1.78	1.82	1.79 ± 0.03	97.47 ± 1.36
2.30	2.13	2.17	2.09	2.13 ± 0.04	92.44 ± 1.71
2.76	2.71	2.69	2.63	2.67 ± 0.04	96.90 ± 1.45

8.2.4 Specificity and selectivity

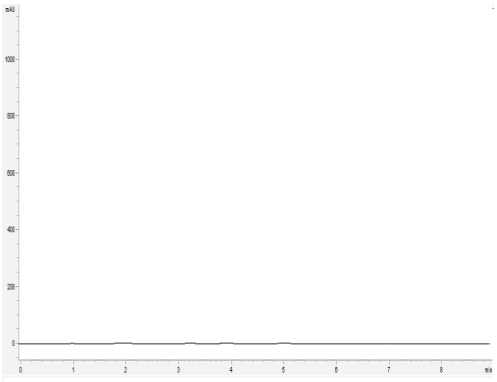
The specificity and selectivity of method was performed using extracted solutions from blank chitosan films and microspheres compared to the extract solutions from the films and microspheres containing diclofenac. The HPLC chromatogram of the extract solutions from the blank chitosan films and chitosan microspheres showed no interference peak, at the retention time of 8.277 min for diclofenac presented in the chromatogram of the diclofenac standard solution (Figure 69).



(A)



(B)



(C)

Figure 69. Chromatograms of (A) a diclofenac standard solution at 20 µg/mL, (B) Extracted solution from blank chitosan film and (C) Extracted solution from blank chitosan microspheres.

8.2.5 Detection and quantification limits

Detection and quantification limits in the HPLC analysis was optimized using diluted diclofenac standard solutions of in a range of 2.0 - 15.0 µg/mL. The limit of detection (LOD) was calculated using parameters of the calibration curve and gave 0.054 µg/mL. The limit of quantification (LOQ) determined based on the parameters of the calibration curve and provided 0.164 µg/mL.

9. Conclusion

Both HPLC and UV visible methods have been investigated for the quantitative determination of diclofenac in chitosan films and microsphere formulations. The analytical procedures were found to be rapid, sensitive, and specific. The accuracy and precision of the method were within the acceptable range according to ICH recommendations. The simplicity of the techniques and the high sensitivity make these techniques particularly attractive for the quantification of diclofenac in both chitosan films and microspheres. These methods are recommended for routine and quality control analysis of the investigated drug in these chitosan formulations.