Thesis for the Degree of Master of Engineering

Fabrication, characterization and anti-microbial activities of poly (ε-caprolactone)/chitosan-caffeic acid composite nano/microfiber mat for wound dressing application

by

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폴리카프로락톤 및 키토산-카페익산 콘쥬게이트 융합형 나노/마이크로 섬유 제작, 특성 및 항균 효능 연구

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by

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A Dissertation

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Abstract

본 연구에서는 창상피복제 제작을 위해 합성을 통해 생리활성효과가 증진된 키토산-카페익산과 우수한 생체적합성, 생분해성 및 적절한 기계적 강도를 보유하고 있는 poly (ε-caprolactone)(PCL)을 혼합한 용액을 이용하여 전기방사를 통해 나노/마이크로 섬유를 제작하였다. 제작된 PCL, 키토산/PCL 및 키토산-카페익산/PCL 나노/마이크로 섬유를 주사전자현미경(SEM)을 통해 관찰한 결과 비드의 형성이 관찰 되지 않음을 확인하여 전기방사를 통한 나노/마이크로 섬유가 이상 없이 제작됨을 확인 하였고, 각각의 나노/마이크로 섬유의 크기를 측정한 결과 1.30 ± 1.07, 1.20 ± 1.22 및 0.94 ± 0.68 μm 를 나타내었다. 추가로 제작된 나노/마이크로 섬유들의 특징들을 관찰하기 위해 퓨리에 변환 적외선 분광기(FTIR) 및 만능 재료시험기(UTM)를 사용하였다. 그 결과, 비록 인간피부와 비교하여 낮은 기계적 강도를 보유하였지만, 전기방사 특성상 높은 기계적 강도를 보유하기 어렵고, 상처주위에 사용되었을 때 높은 하중을 받는 경우가 극히 적음으로 인해, 기계적 강도는 창상피복제의 적용에서 높은 부분을 차지하고 있지 않다. 또한, 제작된 나노/마이크로 섬유들의 세포증식율, 부착형태 및 항균효과에 대한 활성을 알아보고자 NHDF-neo 세포와 그람 양성균인 *Staphylococcus aureus* 를 이용하여 실험을 진행하였다. 그 결과, 키토산-카페익산/PCL 나노/마이크로 섬유의 경우 세포증식률의 증가와 세포독성이 없음을 확인하였다. 또한, 키토산-카페익산/PCL 나노/마이크로 PCL 및 키토산/PCL 나노/마이크로 섬유보다 *S. aureus* 에 대하여 우수한 항균활성을 가짐을 확인하였다. 이러한 결과들을 토대로 키토산-카페익산/PCL 나노/마이크로 섬유는 창상피복제 및 피부조직재생의 적용에 이용될 수 있을 것으로 사료된다.

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List of Abbreviation

AA	Ascorbic acid (vitamin C)
CA	Caffeic acid
CCA	Chitosan-Caffeic acid
CFU	Colony forming units
COS	Chitooligosaccharides
ECM	Extracellular matrix
DDS	Drug delivery system
DMEM	Dulbecco's minimum Eagle's medium
DMSO	Dimethyl sulfoxide
EtOH	Ethanol
FDA	Food and Drug Administration
FTIR	Fourier transform infrared spectroscopy
H_2O_2	Hydrogen peroxide

MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
NHDF-neo	Normal human dermal fibroblasts-neonatal
PBS	Phosphate buffered saline
PCL	Poly (ε-caprolactone)
SEM	Scanning electron microscope
UTM	Universal testing machine

1. Introduction

1.1. Wound healing and wound dressing

1.1.1. Skin

Skin is derived from both ectoderm and mesoderm and average thickness of adult human skin is between 2-3 mm thick (Kusuma, Vuthoori, Piliang & Zins, 2010). It is one of the largest organ in terms of both weight and surface area, and consists of multiple layers such as epidermis, dermis and subcutaneous tissue, and is the most important barrier to prevent toxins and infection entering the body. Among the skin layers, epidermis can be divided in to four layers such as stratum basale, stratum spinosum, stratum granulosum, and stratum corneum. Stratum corneum as outer layer of epidermis consist of dead cells and the other layers consist of viable cells (Hendriks, 1969). Normal human epidermis (a very thin layer: approximately 0.2 mm) composed mainly of keratinocytes (which form 95% of all epidermal cells) with melanocytes (which produce the pigment melanin) and Langerhans's cells (which are part of the skin's immune system) and is a function as barrier against water entry (Barker et al., 1991; Gosain & DiPietro, 2004; Igarashi, Nishino & Nayar, 2007).

The secondary layer of skin between the epidermis and subcutaneous tissues is called as a dermis which forming the bulk of skin (a thick layer: approximately 2 mm) and is composed of many collagen fiber, ground substance and elastin fibers compared with epidermis (Hendriks, 1969; Igarashi, Nishino & Nayar, 2007). These both epidermis and dermis were also called the cutis. The next layer down is the subcutaneous tissues (also

called hypodermis or subcutis) which are used mainly for fat storage and composed of loose fatty connective tissue (Hendriks, 1969).

1.1.2. Wound healing

The wound is defined as damage or loss of the anatomical structure and function of tissue (Velnar, Bailey & Smrkolj, 2009). Skin wound healing is a complex process and requires interactions between the cells, the extracellular matrix (ECM), and the growth factor for tissue regeneration (Schultz & Wysocki, 2009). Skin wound healing process immediately starts after an injury and has been divided into four overlapping but distinct stages such as haemostasis (fibrin clot formation and platelet deposition), inflammation (neutrophils, macrophages, and lymphocytes), proliferation (angiogenesis, fibrogenesis, and re-epithelialization), remodeling (vessel regression and collagen remodeling) (Fig. 1) (Gosain & DiPietro, 2004; Guo & DiPietro, 2010). These healing process is being controlled by the balance between cell proliferation and programmed cell death (Coutinho, Qiu, Frank, Tamber & Becker, 2003). Therefore, interruption of these healing process leads to delayed wound healing and excessive scar formation.

As shown in Fig. 1a, the first stage of wound repair is haemostasis for preventing exsanguination, and its occurs immediately following injury when the platelets adhere to the exposed collagen (Diegelmann & Evans, 2004). Clearly, haemostasis is a major function of blood coagulation and platelet aggregation that it makes the fibrin rich clot which provides as a provisional matrix for migration of various cell such as monocytes, fibroblasts, and endothelial cells into an injured location and sets the next stage for the subsequent healing process (Clark, 2001). Platelets release the clotting factors and provide

a cascade of chemical signals for initiating the chemotaxis of neutrophils, macrophages, smooth muscle cells and fibroblasts, and stimulating the mitogenesis of the fibroblasts and smooth muscle cells (Diegelmann & Evans, 2004).

The second stage of wound repair is inflammation and lasts until about 48h after injury (Fig. 1b) (Gurtner, Werner, Barrandon & Longaker, 2008). Inflammation stage is a major function of immune barrier against invading micro-organisms and can be divided into two phases, an early inflammatory phase and a late inflammatory phase (Velnar, Bailey & Smrkolj, 2009). In the early inflammatory phase (days 1-2), neutrophil granulocytes are attracted to the wound site by a number of chemoattractants such as fragments of ECM protein, transforming growth factor-β, complement components, and formyl-methionyl peptide products from bacteria within 24-48 hours of injury (Enoch & Leaper, 2008). In the late inflammatory phase (days 2-3), monocytes are attracted to the wound site by a number of chemoattractants such as complement, clotting components, fragments of immunoglobulin G, breakdown products of collagen and elastin, and cytokines (Enoch & Leaper, 2008). In particular, macrophages from differentiation of monocytes were the most important cells and involved in the regulation of a number of functions such as production of growth factors (including platelet-derived growth factor, vascular endothelial growth factor, TGF-β, and TGF-α), chemotactic factors (eg, fibronectin), proteolytic enzymes releasing (eg, collagenase), and stimulation of keratinocytes, fibroblasts, and angiogenesis for tissue regeneration (Enoch & Leaper, 2008; Guo & DiPietro, 2010; Li, Chen & Kirsner, 2007).

As shown in Fig. 1c, the third stage of wound repair is new tissue formation and occurs 2-10 days after injury (Gurtner, Werner, Barrandon & Longaker, 2008). In the early part of this stage, keratinocyte proliferate and migrate to the injured dermis, and new blood vessels then formed (Gurtner, Werner, Barrandon & Longaker, 2008; Werner & Grose, 2003). In the later part of this stage, one of the principal reaction to injury is connective tissue cell proliferation such as fibroblast, osteoblast, chondroplasty, odontoblast, and smooth muscle cell (Ross & Tripathi, 1975). Among the connective tissue cell, fibroblasts and myofibroblasts (differentiation cell of fibroblast) interact and produce ECM which mainly consist of collagen in normal human dermis (Gurtner, Werner, Barrandon & Longaker, 2008). Collagen is the main component of ECM that contribute mechanical property of cellular environment such as tissues and organs, and constitutes approximately 30% of total proteins in the animal body (Pati, Adhikari & Dhara, 2010). When tissue defect has occurred by injury, collagen is necessary for repairing of defect, and restoration of anatomic structure, and function of tissue.

Remodeling is the final stage of wound healing process and begins 2-3 weeks after injury and lasts for a year or more (Fig. 1d) (Gurtner, Werner, Barrandon & Longaker, 2008). During this stage, fibrin clot formed in the haemostasis phase is replaced by new tissue known as granulation tissue and various cells such as endothelial cells, macrophages and myofibroblsts undergo programmed cell death known as apoptosis or go out of wound site, which consists mostly of collagen and other ECM protein compare to before skin tissue components (Gurtner, Werner, Barrandon & Longaker, 2008; Li, Chen & Kirsner, 2007). One feature of the wound remodeling phase is ECM remodeling that tensile strength by collagen fiber regain approximately 80% in the granulation tissue compare with

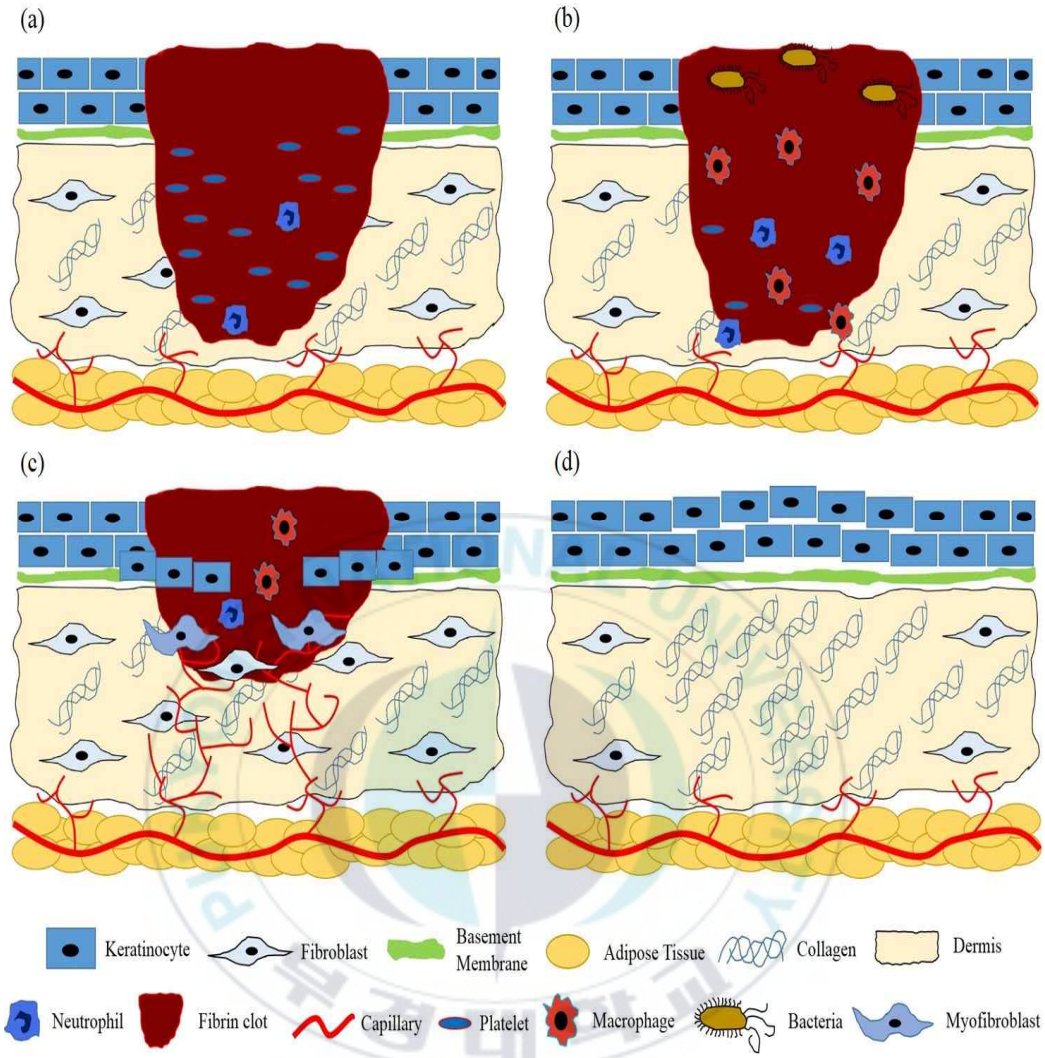

Fig. 1. Wound healing process. There are four classic phase of wound healing: (a) haemostasis, (b) inflammation, (c) new tissue formation, and (d) tissue remodeling.

unwounded tissue that the original strength of the tissue is impossible to regain (Velnar, Bailey & Smrkolj, 2009). To achieve complete wound healing, this four phages have to occur in the suitable sequence and time frame without fail.

1.1.3. Wound dressing

Wound dressing can be divided into three different types such as passive products (gauze and tulle), interactive materials containing polymeric films (hyaluronic acid, hydrogels and foamed covers), and bioactive wound dressing materials (hydrocolloids, alginates, collagens and chitosan) (Singh et al., 2004; Zahedi, Rezaeian, Ranaei-Siadat, Jafari & Supaphol, 2010).

Before 1960s, wound dressing was used to passive products such as gauze and tulle having a minimal role as a common cover in wound healing that this traditional or conventional dressing not suitable for acute and chronic wounds (Zahedi, Rezaeian, Ranaei-Siadat, Jafari & Supaphol, 2010). The interactive materials dressing are occlusive or semi-occlusive and provide a beneficial effect including maintaining of moisture environment and preventing of excessive heat loss on wound site (Singh et al., 2004). Finally, bioactive wound dressing directly affect to wound healing process and new tissue formation by delivery of bioactive compounds such as antimicrobials and growth factors compare with other wound dressing (Boateng, Matthews, Stevens & Eccleston, 2008).

1.1.4. Wound dressing and antimicrobial activity

Wound are susceptible to microbial contamination from both exogenous and endogenous sources (including the nose, skin, mouth, and the gut), and can provide a good environment

(moist, warm, and rich nutritious environment) for the microbial growth and colonization (Bowler, Duerden & Armstrong, 2001; Percival, Bowler & Russell, 2005). After microbial colonization in wounds, microorganism can produce various substances such toxins, proteases and pro-inflammatory molecules, and can delay and fail to the wound healing by causing an excessive and prolonged inflammatory response (Rujitanaroj, Pimpha & Supaphol, 2008). In case of the infection, patient suffers increased several problem such as trauma, treatment costs rise, and more resource demanding for wound healing (Bowler, Duerden & Armstrong, 2001).

Among wound care practitioners, aerobic or facultative pathogens such as Gram positive (*Staphylococcus aureus*) aerobe, Gram negative aerobe (*Pseudomonas aeruginosa*), and beta-hemolytic streptococci are the primary causes of delayed healing and infection in wounds (Bowler, Duerden & Armstrong, 2001). In particular, *S. aureus*, which the Gram-positive bacteria, is the major cause of wound suppuration such as cellulitis, impetigo, and folliculitis, and is currently the most common cause of infections in hospitalized patients that infections or syndromes by *S. aureus* was listed in table 1 (Archer, 1998; Cho et al., 2010).

The anti-bacterial activity in wound dressing application is one of the major important properties for complete wound healing. Thus in the current study, a many of paper have been published about wound dressing by electrospinning with antimicrobial activity for using wound dressing application (Augustine, Kalarikkal & Thomas, 2015; El-Aassar, El-Deeb, Hassan & Mo, 2015; Liao et al., 2015; Thomas, Soumya, Mathew & Radhakrishnan, 2015; Woo, Choi, Choi, Lee & Cho, 2015).

Table 1. Infections or syndromes by Staphylococcus aureus as prominent pathogen (the diseases in boldface are those for which S. aureus is the only or the most common etiologic agent) (Archer, 1998)

Type	Name of a disease
Infection	**Furuncle or carbuncle**
	Impetigo bullosa
	Cellulitis
	Surgical wound infection
	Pyomyositis
	Botryomycosis
	Hospital-acquired bacteremia
	Epidural abscess
	Hospital-acquired pneumonia
	Empyema
	Renal carbuncle
	Septic arthritis
	Brain abscess
	Acute or right-sided endocarditis
	Hematogenous osteomyelitis
	Renal carbuncle
Syndrome	**Toxic shock syndrome**
	Scalded skin syndrome
	Food-borne gastroenteritis

1.2. Electrospinning

Fig. 2 shows that a number of papers for electrospinning have been published and accelerated to paper publication about wound healing and prevent bacterial infections.

Electrospinning process as an old technique is a simple method to produce nano- to micro-sized continuous fibers from various biodegradable polymers and have been used for various industrial and biomedical applications such as tissue engineering, wound dressing and drug delivery system. The fabricated nano- to micro-sized fibers by electrospinning have a high specific surface area-to-volume ratio, interconnectivity, gas permeation and high porosity (Ramakrishna, Fujihara, Teo, Yong, Ma & Ramaseshan, 2006). In addition, it can provide structural similarity between nanometer to micrometer scale substrates and extracellular environment for cell morphology, functionality and cell-cell interactions (Mo, Xu, Kotaki & Ramakrishna, 2004). These properties of electrospinning fiber mats can lead to cell respiration, skin regeneration, moisture retention, removal of exudates, and hemostasis (Rieger, Birch & Schiffman, 2013).

The schematic diagram of the basic setup for electrospinning is shown in Fig. 3. Electrospinning apparatus usually consists of a syringe with stainless-steel needle, a syringe pump, a high voltage power supply and a collector. When the voltage increase to a threshold value, a charged jet of the polymer solution is ejected from the tip of the Taylor cone by overcoming of the surface tension that the jet moves toward a stainless-steel needle to collecting, solvent evaporates and a nano/micro fibrous mat is formed on the collector without solvent (Zeng et al., 2003). This electrospinning process is controlled by various

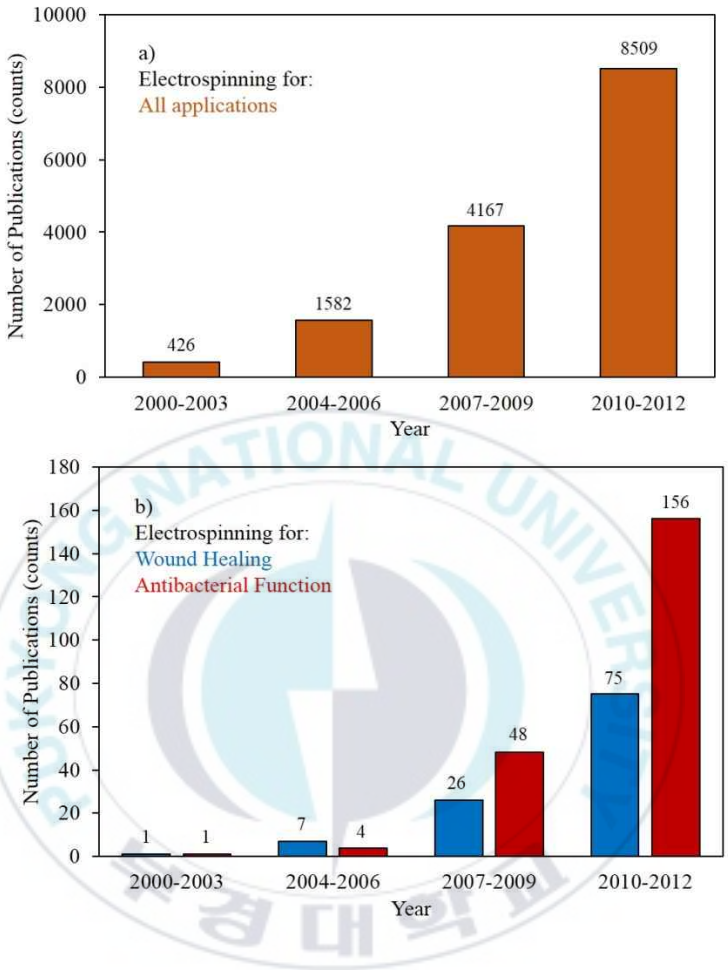

Fig. 2. The using of electrospinning in all applications (a) and specially applications such as wound healing and antibacterial function (b) (Rieger, Birch & Schiffman, 2013).

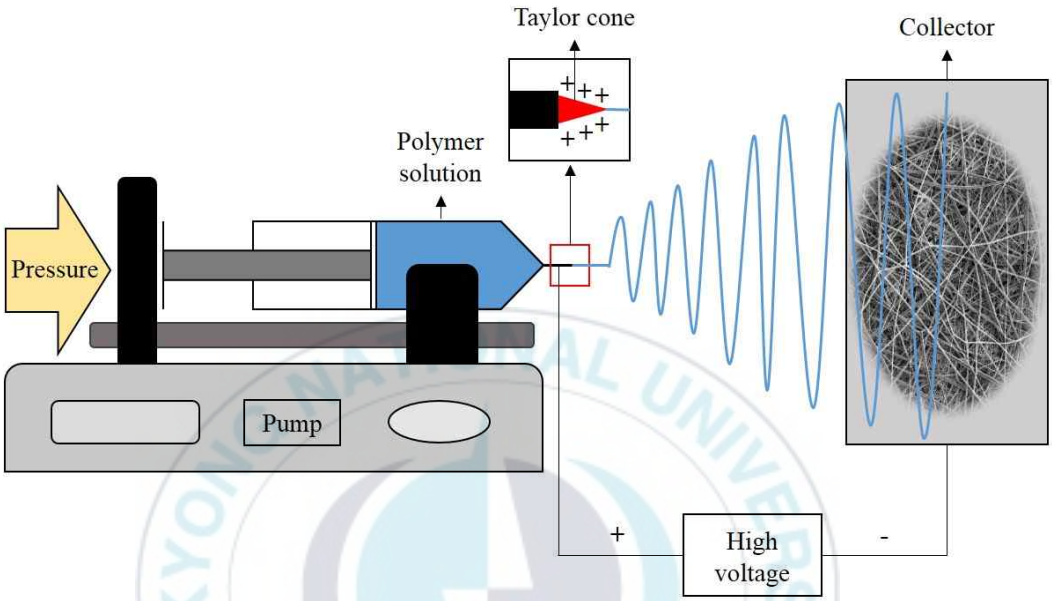

Fig. 3. Schematic diagram of electrospinning components.

parameters such as polymer molecular weight, solution viscosity, conductivity, surface tension, applied electric voltage, tip-to-collector distance, flow rate, temperature, humidity and air velocity (Chronakis, 2005; Geng, Kwon & Jang, 2005).

1.3. Poly (ε-caprolactone)

Polymer can be broadly divided into two parts such as natural polymer and synthetic polymer. Fig. 4 shows the various polymer properties, including biodegradation, integrin binding sites, mechanical properties and physicochemical (hydrophilic or hydrophobic) properties, of commonly used polymer in electrospinning.

The synthetic biodegradable polymers such as polyethylene glycol (PEG), poly(lactic-co-glycolic acid) (PLGA), poly(lactic acid) (PLA) and poly(ε-caprolactone) (PCL) have been used as biomedical applications because they have the characteristics such as structural stability, easily processing and controllability (Cho & Yoo; Jeong et al., 2015; Yeo, Jung & Kim, 2012). These synthetic polymers provided greater advantages than natural materials that they can give various properties such as more predictable lot-to-lot uniformity, more reliable source of raw materials because the polymer materials well know structure and properties and free of concerns of immunogenicity than natural materials such as collagen, gelatin, chitin and alginate (Sabir, Xu & Li, 2009). In particular, among the synthetic polymers, PCL which an aliphatic polyester derived ring-opening polymerization of ε-caprolactone monomer (Fig. 5) has been commonly used as an excellent bioresorbable and biocompatible polymer for use in various medical applications such as tissue engineering and drug delivery system (DDS) (Cheng & Teoh, 2004; Kim, Jung & Kim, 2013). It has good mechanical properties, slower degradation (depending on the molecular

weight, the degree of crystallinity of the polymer, and the conditions of degradation) than other synthetic polymer, low glass transition and melting temperatures (T_g = -50°C, T_m = 60°C) and was approved by the US Food and Drug Administration (FDA) for use clinical application in human body (López-Rodríguez, López-Arraiza, Meaurio & Sarasua, 2006; Labet & Thielemans, 2009).

Dissolving type of PCL polymer in various solutions can be classified into three types such as soluble, low soluble and insoluble solution, and is detailed in Table 2. Among the PCL properties, one of the rare property is miscible polymer with other various polymers such as poly (vinyl chloride), poly (styrene-acrylonitrile), poly (acrylonitrile butadiene styrene), poly (bisphenol-A) and other polycarbonates, nitrocellulose and cellulose butyrate, and is mechanically compatible with polypropylene, polyethylene, natural rubber, poly (vinyl acetate), ethylene/propylene rubber (Lepoittevin et al., 2003).

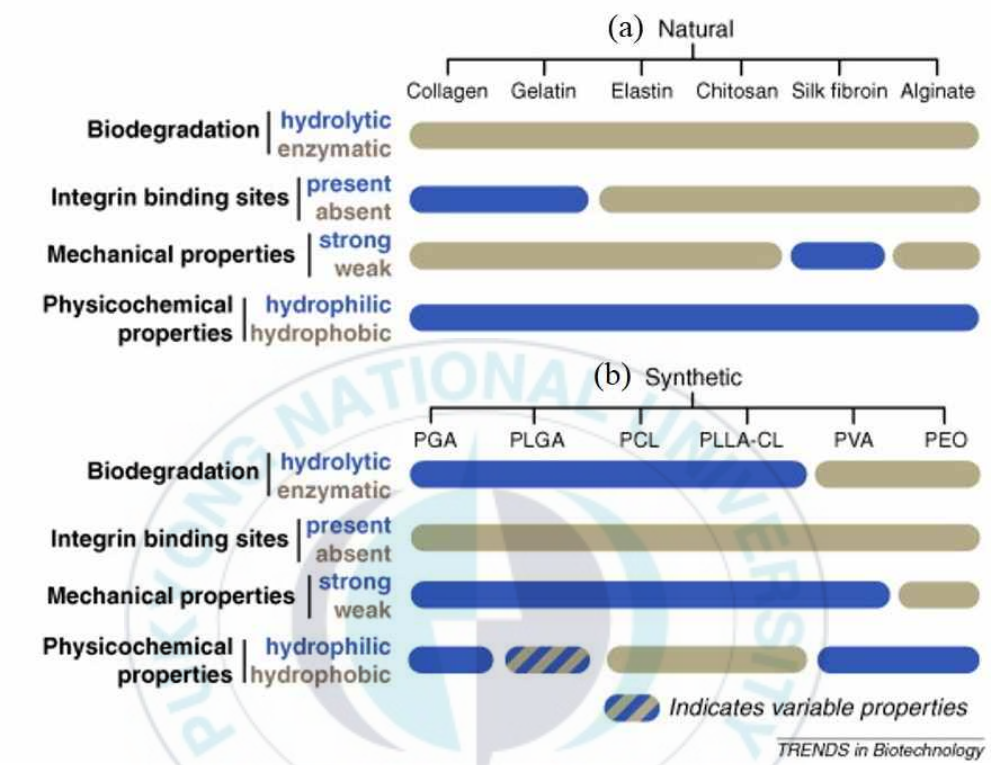

Fig. 4. Properties of commonly used various polymers in electrospinning filed (Gunn & Zhang, 2010). (a) Natural polymer and (b) synthetic polymer.

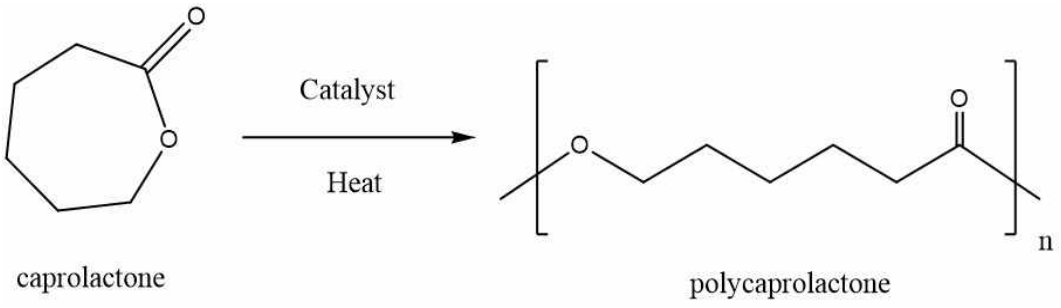

Fig. 5. Ring opening polymerization of caprolactone to polycaprolactone.

Table 2. Dissolving of PCL polymer in various solution at room temperature (Labet & Thielemans, 2009).

Dissolve	Solvent name
Soluble	Chloroform, dichloromethane, carbon tetrachloride, benzene, toluene, cyclohexanone and 2-nitropropane
Low soluble	Acetone, 2-butanone, ethyl acetate, dimethylformamide and acetonitrile
Insoluble	Alcohol, petroleum ether, diethyl ether and water

1.4. Chitosan

Chitin derived from main sources such as crabs and shrimps known as crustaceans is second most abundant natural polysaccharide of linear N-acetylglucosamine repeating units (Fig. 6) after cellulose on earth and component of the cell walls of fungi and yeast, the exoskeletons of arthropods and insects (Chen, 2013; Pillai, Paul & Sharma, 2009).

Chitosan, which degree of deacetylation ranges from 60% to 100%, derived from deacetylate of naturally occurring chitin is linear and aminopolysaccharide consisting of linear β-1,4-linked D-glucosamine and *N*-acetyl-D-glucosamine repeated unit (Fig. 7), and molecular weight is between 3800 to 20,000 Daltons (Anitha et al., 2014; Chen, 2013; Cheung, Ng, Wong & Chan, 2015).

It has been widely applied for various biological activity including antimicrobial activity (Lee, Jeong, Kim, Lee, Ahn & Je, 2009), anti-oxidant (Je & Kim, 2006), anti-inflammation (Cho, Lee, Kim, Ahn & Je, 2011), antitumor (Qin, Du, Xiao, Li & Gao, 2002), anti-HIV (Dev et al., 2010), anticoagulant (Park, Je, Jung, Ahn & Kim, 2004) and anti-fungal activity (Lopez-Moya et al., 2015), and biomedical applications such as bone tissue engineering (Frohbergh et al., 2012; Lee, Jin, Jang, Jung & Kim, 2013), skin tissue engineering (Shalumon, Sathish, Nair, Chennazhi, Tamura & Jayakumar, 2012), neural tissue engineering (Guan, Zhang, Lin, Liu, Ma & Cui, 2013), cartilage tissue engineering (Park, Choi, Hu & Lee, 2013), vascular tissue engineering (Du et al., 2012), cardiac tissue engineering (Martins, Eng, Caridade, Mano, Reis & Vunjak-Novakovic, 2014) and corneal tissue engineering (Ozcelik, Brown, Blencowe, Daniell, Stevens & Qiao, 2013) due to its

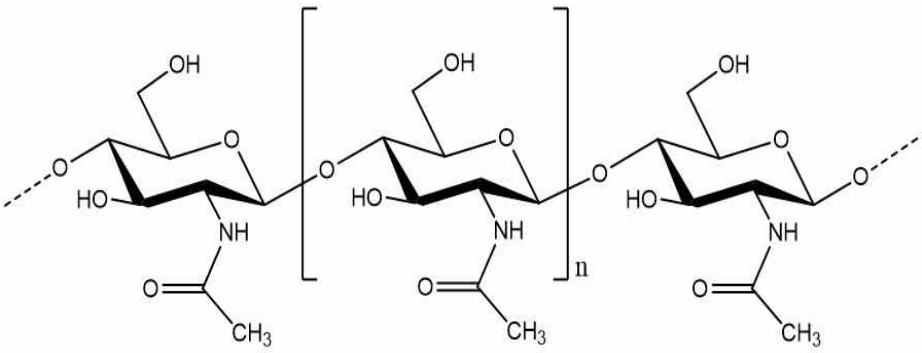

Fig. 6. Chemical structure of chitin.

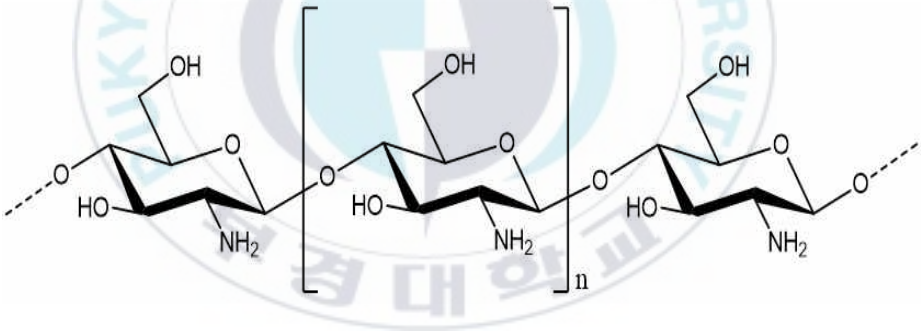

Fig. 7. Chemical structure of chitosan.

several properties such as biodegradability, biocompatibility, non-toxicity, low allergencity and biodegradability (Chandika et al., 2015; Cheung, Ng, Wong & Chan, 2015).

1.4.1. Chitosan-antioxidant compound conjugates

Chemical modification of natural polymers is one of the methods for production of new biomaterials and bioactive agents with improved or specific properties. A number of papers have been grafted onto chitosan backbone and investigation of properties that conjugated chitosan listed in Table 3. The results show that chitosan conjugates improve biological activity compare with non-grafted chitosan. Grafted onto chitosan backbone methods can be divided into three kind of methods such enzyme catalyzed coupling reaction (Aljawish et al., 2012), carbodiimide mediated coupling reaction (Wang, Liu, Jiang & Zhang, 2007) and free radical-induced grafting method (Lee, Woo, Ahn & Je, 2014).

Among the grafted onto chitosan backbone methods, free radical-induced grafting reaction, which ascorbic acid (AA) and hydrogen peroxide (H_2O_2) redox system, proceed in room temperature without generation of toxic reaction products and antioxidant degradation by lower temperature (Curcio et al., 2009). Briefly, AA reacts with H_2O_2 and produces hydroxyl radical ($^{\bullet}OH$) like Fenton reaction, and initiates conjugation as shown in Fig. 8 (a, b). The $^{\bullet}OH$ resulting from AA oxidation by H_2O_2 can attack to H-atoms in α-methylene or hydroxyl groups of hydroxylmethylene group or amino group of the chitosan as shown Fig. 8 (c) (Mun et al., 2008). At those sites, the grafting of the antioxidant compounds can occur at room temperature as shown Fig. 8 (d).

Table 3. Conjugate method and biological activity of conjugated chitosan (continued).

Chitosan Mw[a]	Compound	Grafting method	Biological activity	Reference
~310 kDa	Gallic acid	AA[b]-H_2O_2[c] redox system	Antioxidant activity	(Cho, Kim, Ahn & Je, 2011b)
~310 kDa	Gallic acid	AA-H_2O_2 redox system	Acetylcholinesterase inhibition	(Cho, Kim, Ahn & Je, 2011a)
~310 kDa	Phloroglucinol	AA-H_2O_2 redox system	Antioxidant activity Antimicrobial activity	(Lee, Cho & Je, 2013)
~310 kDa	Phloroglucinol	AA-H_2O_2 redox system	Antioxidant activity Tyrosinase inhibition	(Woo & Je, 2013)
250 kDa	Caffeic acid Gallic acid Ferulic acid	AA-H_2O_2 redox system	Antioxidant activity	(Liu, Lu, Kan, Tang & Jin, 2013)
NM[d]	Caffeic acid Ferulic acid	AA-H_2O_2 redox system	Antioxidant activity	(Liu, Wen, Lu, Kan & Jin, 2014)
~310 kDa	Caffeic acid Ferulic acid Sinapic acid	AA-H_2O_2 redox system	Antioxidant activity Antimicrobial activity	(Lee, Woo, Ahn & Je, 2014)

Chitosan Mw	Compound	Grafting method	Biological activity	Reference
421.5 kDa	Gallic acid Caffeic acid	Laccase-catalyzed oxidation	Antioxidant activity Antimicrobial activity	(Božič, Gorgieva & Kokol, 2012)
310-375 kDa	Ferulic acid Ethyl ferulate	Laccase-catalyzed oxidation	Antioxidant activity	(Aljawish et al., 2012)
544 kDa	Caffeic acid	EDC[e] mediated coupling reaction	Antioxidant activity	(Aytekin, Morimura & Kida, 2011)
250 kDa	Protoporphyrin IX	EDC mediated coupling reaction	Anticancer activity	(Lee et al., 2011)
~200 kDa	Ferulic acid	EDC mediated coupling reaction	Antioxidant activity	(Woranuch & Yoksan, 2013)

[a]Molecular weight, [a]Ascorbic acid, [b]Hydrogen peroxide, [c]Not mention, [d]1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide.

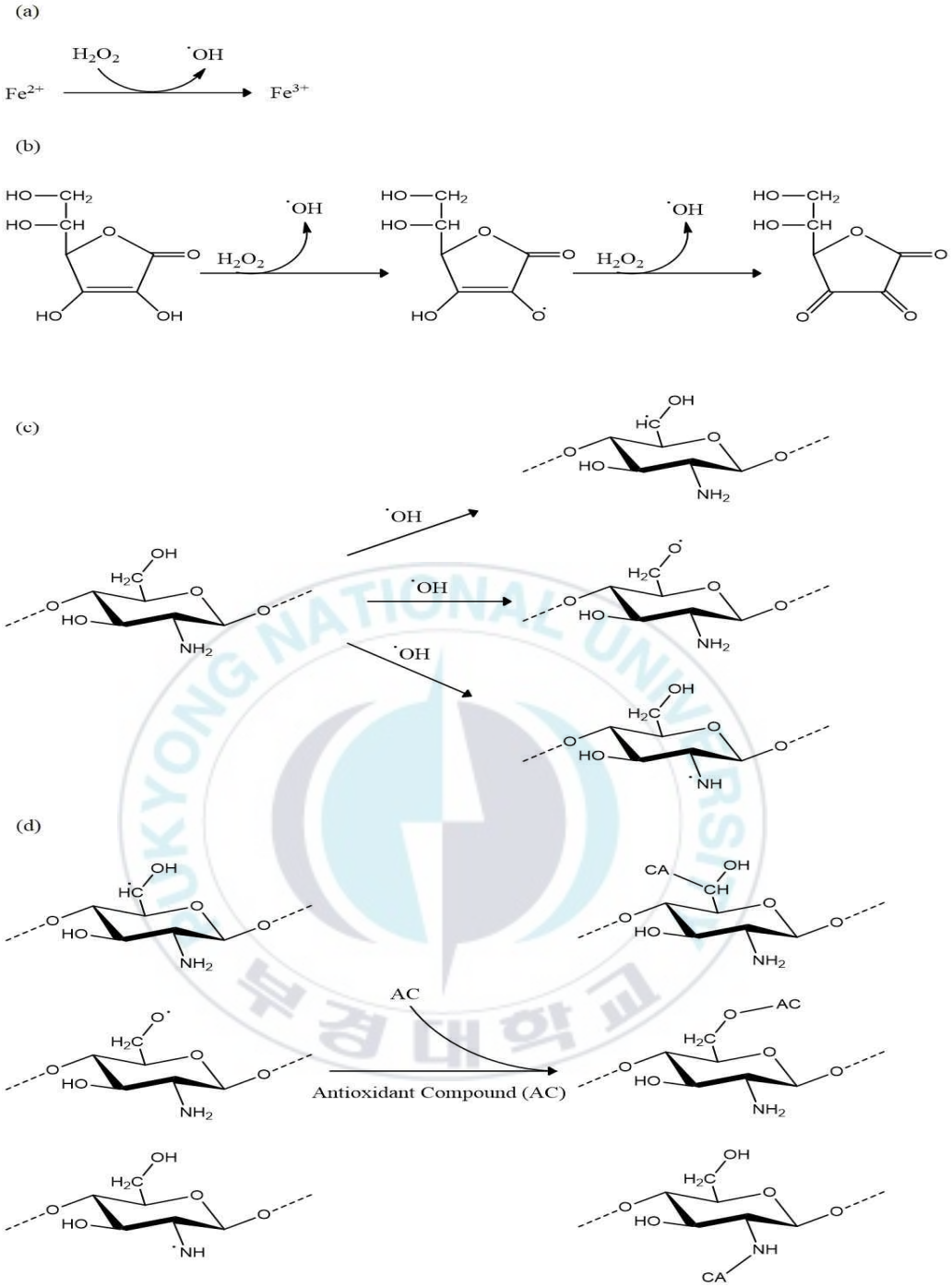

Fig. 8. Fenton reaction (a), ascorbic acid oxidation by H_2O_2 (b) and insertion of antioxidant compound in chitosan by redox system (c, d).

1.5. Caffeic acid

Hydroxycinnamic acid are secondary plant metabolites and is the major class of phenolic compounds, which one of the major hydroxycinnamic acids is caffeic acid (3,4-dihydroxycinnamic acid) (Chen & Ho, 1997; Gülçin, 2006). Caffeic acid is derived from chlorogenic acid (3-caffeoyl-D-quinic acid), which is an ester formed between caffeic acid and quinic acid, and is found as antioxidant in plants, fruits and vegetables (Kono et al., 1997; Sato et al., 2011) (Fig. 9). This caffeic acid have been shown to possess diverse biological activity such as anti-oxidant (Gülçin, 2006), anti-cancer (Prasad, Karthikeyan, Karthikeyan & Reddy, 2011), anti-bacterial and anti-fungal effects (Aziz, Farag, Mousa & Abo-Zaid, 1997), anti-hepatitis B virus activity (Wang et al., 2009), and anti-mutagenic activity (Yamada & Tomita, 1996).

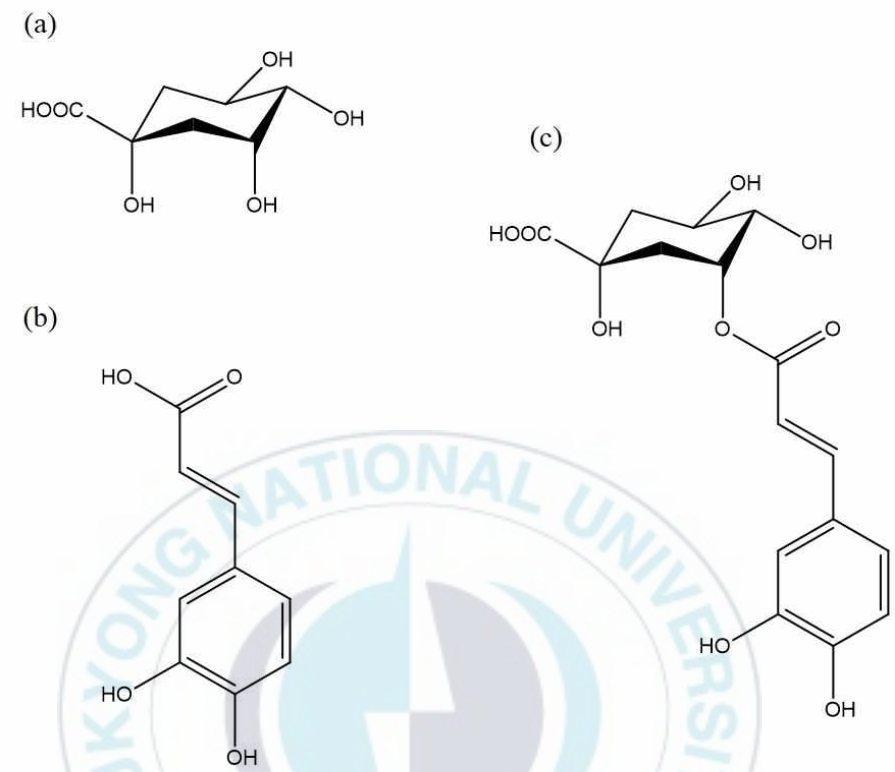

Fig. 9. Chemical structure of (a) quinic acid, (b) caffeic acid and (c) chlorogenic acid.

1.6. Experimental design

The aim of the present study was to fabricate nano/microfiber mat with antimicrobial activity. Fig. 10 showed schematic illustration of the experimental design for chitosan-caffeic acid loaded PCL nano/microfiber mat. First, to fabricate the chitosan-caffeic acid

loaded PCL nano/microfiber mat, we synthesized the chitosan-caffeic acid conjugate by ascorbic acid-hydrogen peroxide redox system. And then, synthesized chitosan-caffeic acid blended with 12% PCL solution, and fabricated the chitosan-caffeic acid loaded PCL nano/microfiber mat. After fabrication of nano/microfiber mat, to determine the characterization of fabricated fiber mat, we carried out SEM, FT-IR and UTM. In addition, we indicated the *In vivo* experimentation such as cell proliferation by MTT assay, cell morphology by SEM. Finally, we carried out antimicrobial activity against *Staphylococcus aureus*.

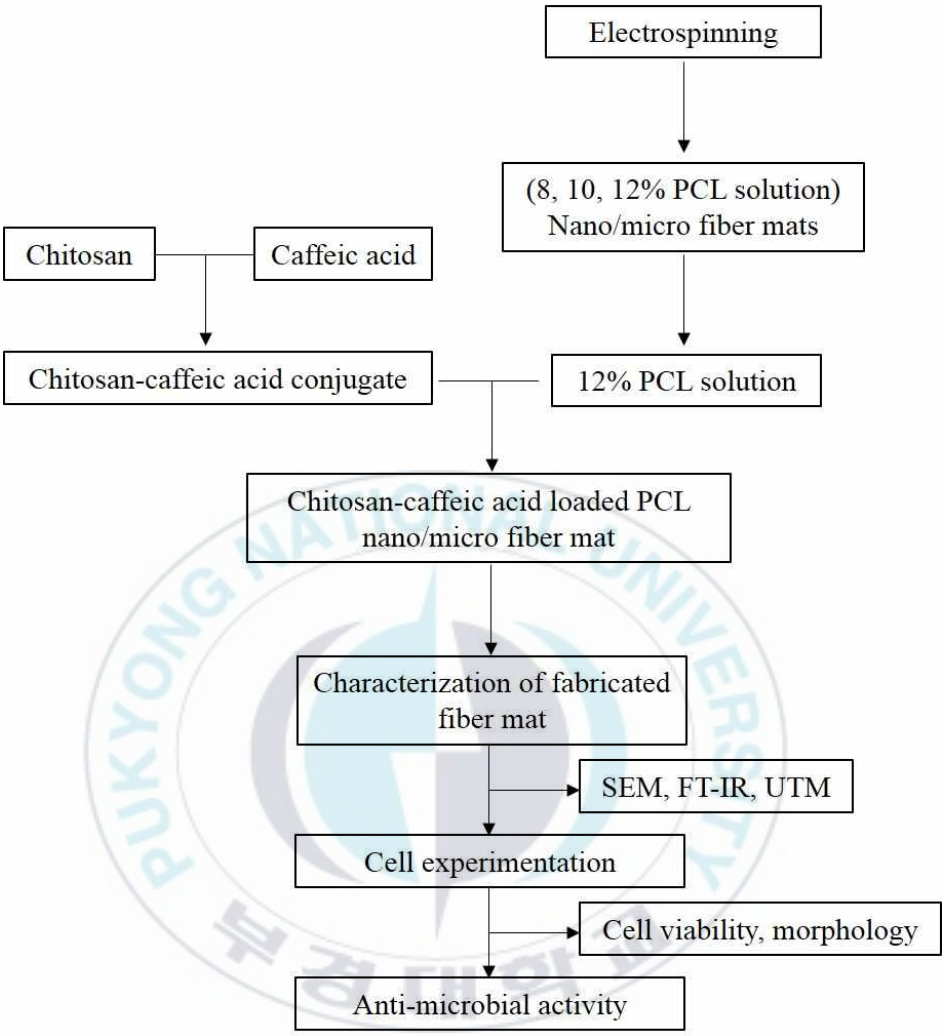

Fig. 10. Schematic illustration of the experimental design.

2. Materials and methods

2.1. Materials

Chitosan (average Mw 310 kDa and 90% degree of deacetylation) was donated from Kitto Life Co. (Seoul, Korea). Poly (ε-caprolactone) (PCL; Mw 80,000), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), chloroform, caffeic acid, and methanol were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS; pH 7.4), trypsin-EDTA, and penicillin/streptomycin were purchased from Gibco-BRL (Gaithersburg, MD, USA). All other chemicals and reagents used in this study were of analytical grade. All aqueous solutions were prepared using triply-distilled water.

2.2. Preparation of chitosan-caffeic acid (CCA) conjugates

CCA conjugate was prepared according to previous method (Lee, Woo, Ahn & Je, 2014). 0.25 g of chitosan was dissolved in 25 ml of 2% acetic acid, and 0.5 ml of 1.0 M hydrogen peroxide containing 0.054 g of ascorbic acid was then added. After 30 min, caffeic acid (CA) was added to the mixture with the following molar ratios of chitosan repeat unit to CA; 1:0.1. The mixture was allowed to rest at room temperature for 24 h, and was then dialyzed to remove the unreacted CA. The unmodified chitosan was also prepared without the addition of CA. In the previous study, conjugated CCA was analyzed by ^{1}H NMR spectrometer as shown in Fig. 11.

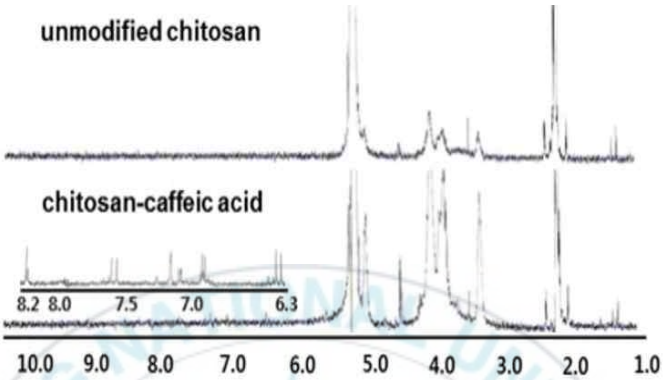

Fig. 11. ^{1}H NMR spectra of chitosan-caffeic acid conjugate and non-conjugate chitosan (Lee, Woo, Ahn & Je, 2014).

2.3. Optimal condition of nano/microfiber mat and fabrication of PCL/chitosan-caffeic acid nano/microfibers

The PCL nano/microfibers mats were fabricated using electrospinning. PCL solution was prepared by dissolving 3.2, 4.0 and 4.8 g of PCL in 40 g of a solvent mixture of 75 wt% chloroform and 25 wt% methanol (8, 10 and 12% PCL solution). The polymer solution was placed in a 5-ml syringe with a 23 G stainless needle. The feed rate of the solution (0.75 ml/h) was precisely controlled by a syringe pump system (KDS 100; KD Scientific). The applied voltage was 8.5 kV and 11 kV. The collection plate of aluminum foil was located at a distance of around 15 to 27 cm from the stainless needle tip. And then, to fabricate sample-loaded nano/microfiber mats, the solutions used were pure PCL (P), PCL with 0.1% chitosan (PC), and PCL with 0.1% chitosan-caffeic acid (PCCA) at several condition (Fig. 12). Table 4 shows the detailed condition for fabrication of nano/microfiber mats.

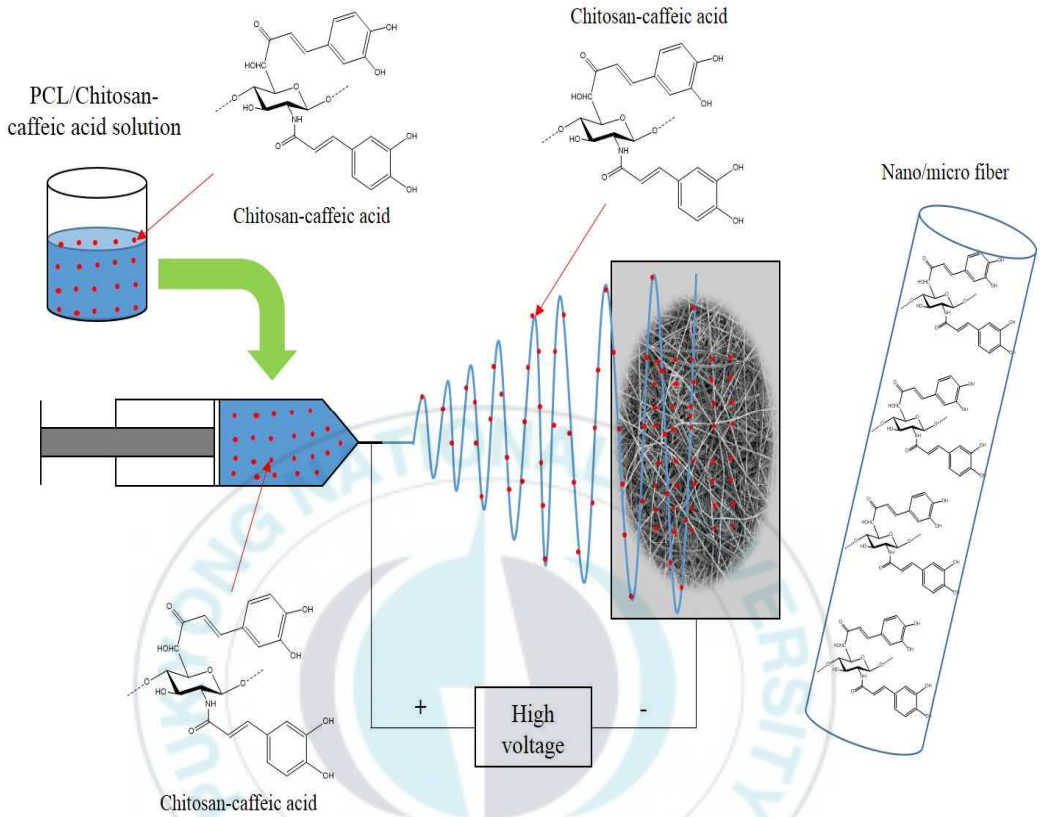

Fig. 12. Schematic illustration of the process of fabrication of chitosan-caffeic acid loaded PCL nano/microfiber mat.

Table 4. Condition for fabrication of electrospinning nano/microfiber mats.

Solution (%)	Voltage (kV)	Distance (cm)	Bead formation
8	7.2	15	+++
10	7.2	15	+
	8.5	18	+
	9.6	21	+
	10.5	24	+
	11	27	+
12	7.2	15	ND[a]
	8.5	18	ND
	9.6	21	ND
	10.5	24	ND
	11	27	ND

[a]Not detection.

2.4. Scaffold characterization

2.4.1. Microstructural evaluation

The structural morphology of the fabricated nano/microfiber membrane were examined using scanning electron microscope (SEM, Tescan, Czech, VEGA II LSU) at 15 kV. The diameter of nano/microfiber membrane were measured from the SEM image using image analysis software (Image J, National Institutes of Health, USA).

2.4.2. Fourier-transform infrared (FT-IR) spectroscopy

FT-IR spectroscopy (Perkin Elmer, USA) data were collected from pure P, PC, and PCCA fiber mats to determine the functional groups of fabricated fiber mats. The IR spectra represent the average of 30 scans between 500 and 4000 cm^{-1} at a resolution of 4 cm^{-1}.

2.4.3. Tensile test

Mechanical properties of fabricated nano/microfiber membrane were determined with a universal testing machine (Top-tech 2000; Chemilab, Suwon, South Korea). Samples measuring 5 mm x 15 mm were prepared, and the mechanical data were acquired in four independent experiments. The samples were stretched to failure at a stretching speed of 0.5 mm/s at room temperature.

2.5. Cell experimentation

2.5.1. Cell culture

Cell culture studies on nano/microfiber membrane were performed using NHDF-neo cell line in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin. NHDF-neo cells harvested from 80% confluence were used to seed onto fiber membrane to investigate the cell viability of the fabricated fiber membrane (P, PC, and PCCA). Before cells were seeded, fabricated fiber membrane were sterilized with 70% EtOH and UV light, and then placed in culture medium and incubated in 5% CO_2 at 37 ºC.

2.5.2. Cell viability and proliferation

Cell viability was determined by the MTT assay. The fabricated fiber membrane were punched (15 mm in diameter) and placed 24 cell culture plate. The NHDF-neo cells were collected by trypsin-EDTA treatment, seeded onto the fabricated fiber membrane, and incubated at 37 ºC in and atmosphere of 5% CO_2. After incubation, each cell seeded fiber membrane were washed with PBS and re-incubated with culture medium and MTT solution (1 mg/ml in PBS) at 37 ºC for 4 h. After 4 h, the solution was aspirated and re-incubated with DMSO at 37 ºC for 30 min. After 30 min, the absorbance at 570 nm was measured using a microplate reader (Gen 5^{TM} ELISA BioTek, USA).

2.5.3. Cell morphology

The fabricated fiber membrane were punched (15 mm in diameter) and placed 24 cell culture plate. The NHDF-neo cells were collected by trypsin-EDTA treatment, seeded onto

the fabricated fiber membrane, and incubated at 37 ºC in and atmosphere of 5% CO_2. Cell morphology was observed using SEM. After 1 and 7 days of incubation, cell attached fiber membrane were washed three time with PBS (pH 7.4) and fixed with 2.5% glutaraldehyde in PBS for 5 h at 4 ºC and thoroughly washed with PBS. Thereafter, each fiber membrane were dehydrated with an ascending series of EtOH (50%, 70%, 80%, 90%, 95%, 99%, 100%) and subsequently samples were freeze dried and observed under SEM.

2.6. Antimicrobial activity

The antibacterial activity of the mats against the Gram-positive bacterium *Staphylococcus aureus* was assessed by a viable cell-counting method. Upon appropriate dilution with sterilized 0.9% saline solution, a culture of about 10^5 CFU/ml was prepared and used for antibacterial testing. The *S. aureus* suspension was incubated in the presence of the nano/microfiber membrane at 37 ºC. At various exposure times (0, 30, 60, 120 and 240 min) aliquots were taken from the bacterial suspension and several decimal dilutions were made. The several decimal dilutions were quickly spread on the nutrient agar and incubated at 37 ºC for 24 h. The surviving microorganisms were counted by the spread-plate method in triplicate for each experiment. The number of the surviving cells was determined as colony forming units (CFU).

2.7. Statistical analysis

All quantitative data are presented as means ± standard deviation (SD) with least three individual experiments that were conducted using fresh reagents. Differences between the means of each group were assessed by one-way analysis of variance (ANOVA) followed

by Duncan's test using the statistical soft-ware, PASW Statistics 21.0 (SPSS Inc., Chicago, IL, USA). The differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Morphological analysis of fabricated nano/microfibers

The morphology and fiber diameter of electrospinning nano/microfiber mats were examined by scanning electron microscope (SEM). Fig. 13 shows SEM images of electrospinning at different poly (ε-caprolactone, PCL) concentrations (8, 10 and 12%) in chloroform/methanol solution. As shown Fig. 13a, at low PCL concentration (8%) in the form beads is observed like electrospray rather than electrospinning. The presence of bead formation have been seen, indicating that the polymer fiber was not enough dry time to reaching the collector. However, increasing the PCL polymer concentration yielded uniform fibers with (Fig. 13b) or without (Fig. 13c) few bead formation compare with 8% PCL solution. In addition, 12% PCL solution have been seen increased fiber diameter compare with 8 and 10% PCL solution. Therefore, we have used 10 and 12% PCL solution as the next experiment cause of the relatively small then bead formation with 8% PCL solution.

Fig. 14 shows SEM images of 10% and 12% PCL electrospinning at different needle-to-collector such as 18, 21, 24 and 27, and voltage such as 8.5, 9.6, 10.5 and 11 kV. As shown in Fig. 14 (a, c, e and g), 10% PCL nano/microfiber mats in the form beads are little observed more than 12% PCL fiber mat at different condition (voltage and needle-to-collector distance). However, 12% PCL nano/microfiber mats showed bead free morphology at different condition (Fig. 14b, d, f and h). In addition, 12% PCL nano/microfiber mats did not significantly change the morphology in different condition at

voltage (8.5 to 11 kV) and needle-to-collector distance (18 to 27 cm). Therefore, we have used 12% PCL solution as the next experiment for fabricating of sample loaded nano/microfiber mats.

Fig. 15 shows morphology of the PCL, 0.1% w/w chitosan loaded PCL and 0.1% w/w chitosan-caffeic acid loaded PCL nano/microfiber mats in 500X (Fig. 15a, b and c) and 3000X (Fig. 15d, e and f), and each fabricated fiber diameter. Chitosan-loaded PCL and chitosan-caffeic acid loaded PCL nano/microfiber mats showed bead free morphology and not significantly changed the fiber diameter (1.20±1.22 and 0.94±0.68 μm) compare with non-loaded PCL nano/microfiber mat (1.30±1.07 μm) as shown in Fig. 15 (g, h and i).

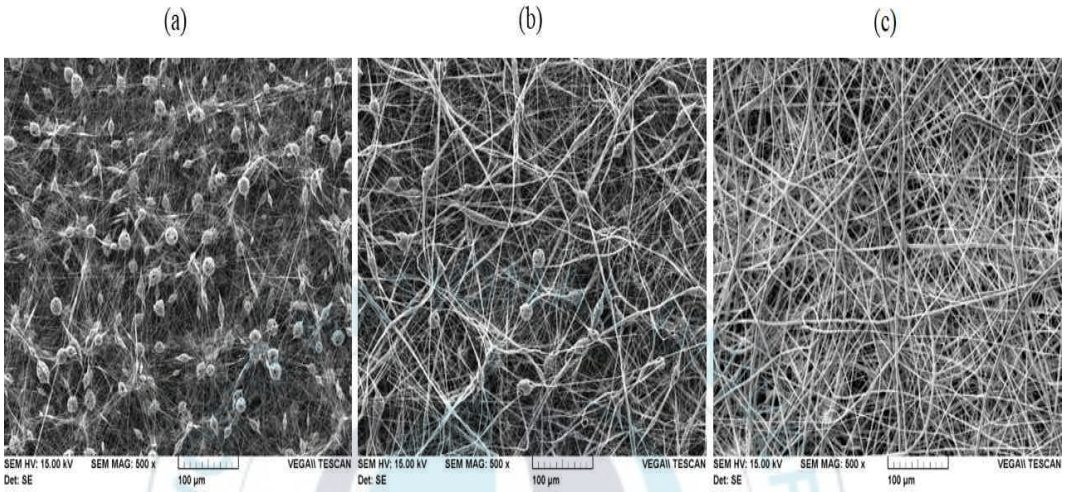

Fig. 13. SEM micrographs of electrospinning PCL fiber mats showing the morphology change at different PCL polymer concentration. (a) 8% PCL solution, (b) 10% PCL solution and (c) 12% PCL solution. Voltage = 7.2 kV, distance to collector = 15 cm.

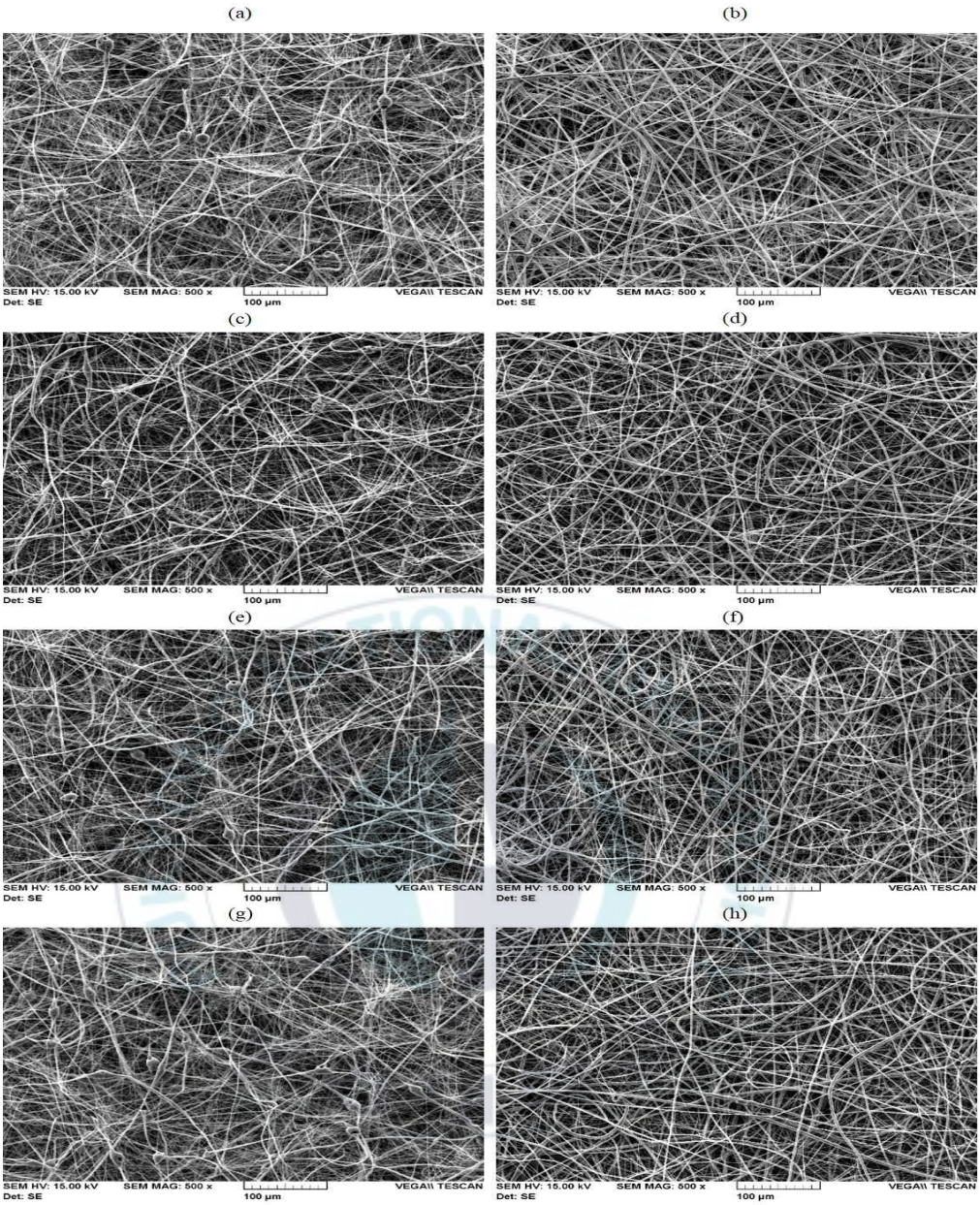

Fig. 14. SEM micrographs of electrospinning PCL polymer membrane showing the morphology change at different condition. 10% PCL solution (A, 18 cm, 8.5 kV; C, 21 cm, 9.6 kV; E, 24 cm, 10.5 kV; G, 27 cm, 11 kV) and 12% PCL solution (B, 18 cm, 8.5 kV; D, 21 cm, 9.6 kV; F, 24 cm, 10.5 kV; H, 27 cm, 11 kV)

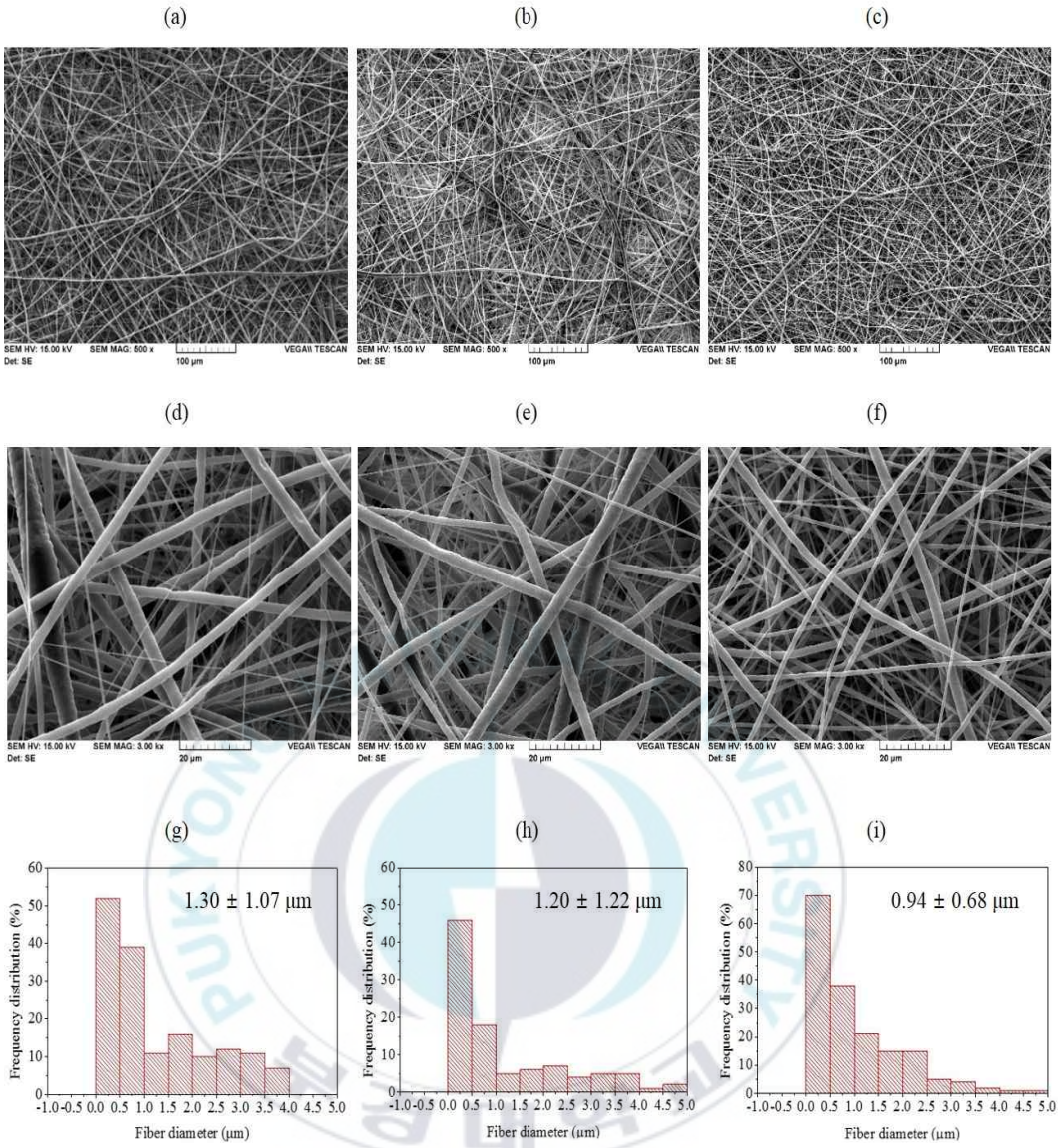

Fig. 15. SEM micrographs of fabricated nano/microfiber mats showing the morphology. (a; 500X, d; 3000X, g; fiber diameter) non-loaded PCL (P) fiber mat, (b; 500X, e; 3000X, h; fiber diameter) chitosan-loaded PCL (PC) fiber mat and (c; 500X, f; 3000X, i; fiber diameter) chitosan-caffeic acid PCL (PCCA) fiber mat.

3.2. FT-IR

Fig. 16 shows the FT-IR reflection spectra for the non-loaded PCL, chitosan-loaded PCL and chitosan-caffeic acid loaded PCL nano/microfiber mats. The peaks located at 2941, 2864, and 1723 cm^{-1} of non-loaded PCL nano/microfiber mat were assigned to the stretching of asymmetric CH_2, stretching of symmetric CH_2, and stretching of C=O, respectively. Also, the peaks located at 1294, 1241 and 1188 cm^{-1} were assigned to the stretching of C-O and C-C, stretching of asymmetric C-O-C, and stretching of symmetric C-O-C, respectively (Gautam, Dinda & Mishra, 2013). In the fabricated chitosan and chitosan-caffeic acid loaded PCL nano/microfiber peak, this PCL peak was also absorbed. In the FT-IR spectra of chitosan-caffeic acid PCL nano/microfiber mat, the characteristic peak at 3441 cm^{-1} of hydroxyl end-group increased by adding of chitosan-caffeic acid conjugate.

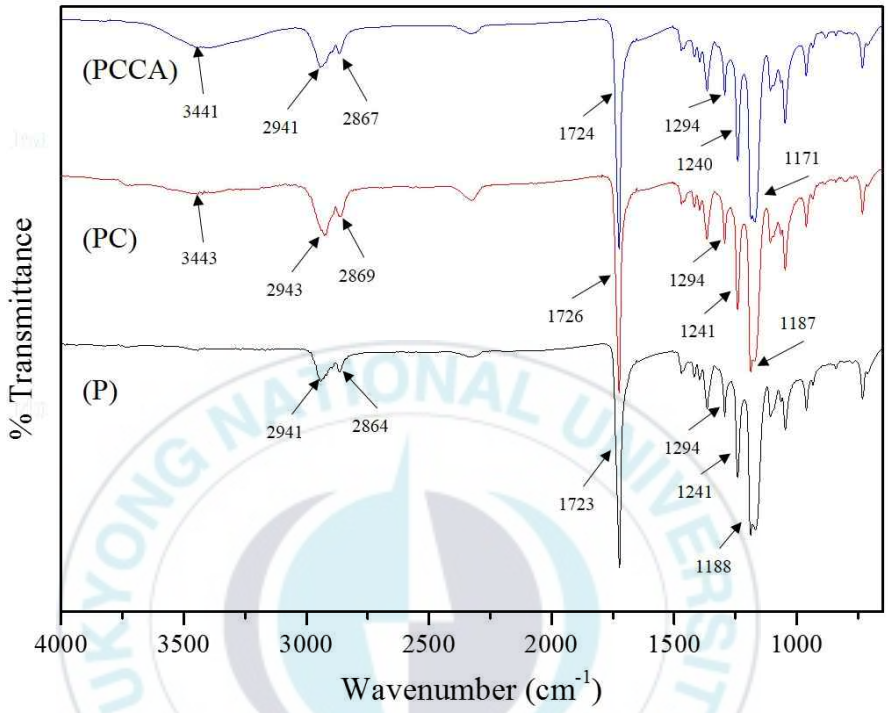

Fig. 16. FT-IR spectra of non-loaded PCL (P), chitosan-loaded PCL (PC) and chitosan-caffeic acid (PCCA) nano/microfiber mats.

3.3. Mechanical properties

The mechanical properties of fabricated nano/microfiber mats were collected using universal testing machine. Fig. 17 shows the stress-strain curves of different electrospinning nano/microfiber mats under tensile loading. The stress-strain curves is non-linear curve cause The tensile strength of the non-loaded PCL, chitosan-loaded PCL and chitosan-caffeic acid PCL nano/microfiber mats was determined 1.95±0.52, 2.17±0.21 and 2.67±0.17 MPa, respectively. Also, the tensile modulus of fabricated nano/microfiber mats was calculated 4.45±1.35 (non-loaded PCL), 6.41±0.31 (chitosan-loaded PCL) and 9.64±1.41 (chitosan-caffeic acid loaded PCL) MPa, respectively. The tensile modulus was calculated through Secant modulus of elasticity because this stress-strain curve is non-linear. Table 5 shows the detailed other mechanical properties such as ultimate strain of the fabricated nano/microfiber mats compare with human skin.

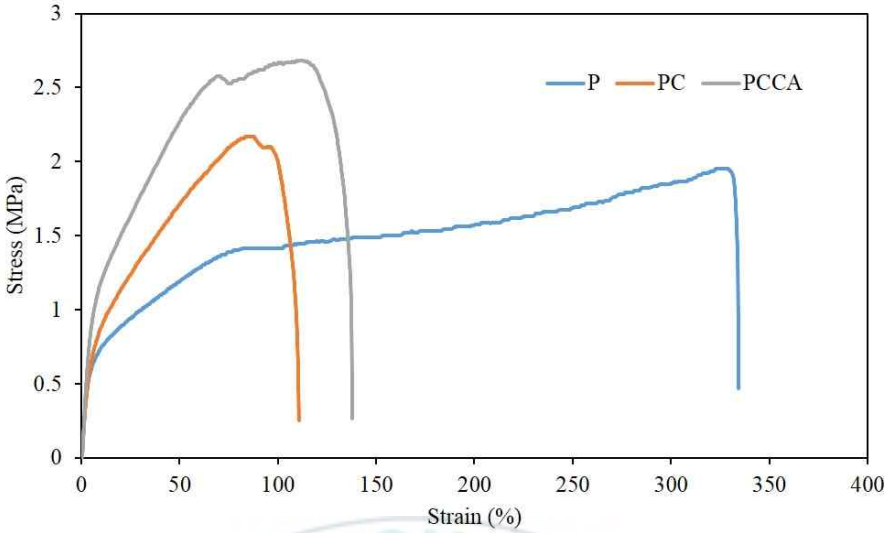

Fig. 17. Stress-strain curve of non-loaded PCL (P), chitosan-loaded PCL (PC) and chitosan-caffeic acid PCL (PCCA) nano/microfiber mats.

Table 5. Tensile properties of the fabricated nano/microfiber mats.

Sample type	P[a]	PC[b]	PCCA[c]	Human[d]
Tensile modulus (MPa)	4.45 ± 1.35	6.41 ± 0.31	9.64 ± 1.41*	15-150
Ultimate tensile strength (MPa)	1.95 ± 0.52	2.17 ± 0.21	2.69 ± 0.17*	5-30
Ultimate strain (%)	326.91 ± 36.15	77.45 ± 9.93*	126.66 ± 19.14*	35-115

[a]Non-loaded PCL, [b]chitosan-loaded PCL, [c]chitosan-caffeic acid PCL, [d]Human skin (Jin, Prabhakaran, Kai, Annamalai, Arunachalam & Ramakrishna, 2013), *$P < 0.05$.

3.4. Cell proliferation

The proliferation rate of NHDF-neo cells on electrospinning nano/microfiber mats after 1, 3 and 7 days was determined by MTT assay (Fig. 18). It was observed that NHDF-neo cells on all the fabricated nano/microfiber mats increased during the cell culture for 7 days. The results suggested that the NHDF-neo cells were promoted cell growth on chitosan or chitosan-caffeic acid containing nano/microfiber mats and did not shown cytotoxic effects on fabricated nano/microfiber mats. The percentage increase in the rate of proliferation from day 1 to 7 days on non-loaded PCL, chitosan-loaded PCL and chitosan-caffeic acid loaded PCL nano/microfiber mats was found to be 66%, 76% and 83%, respectively. Results of NHDF-neo cells proliferation assay suggested that the electrospinning chitosan-caffeic acid loaded PLC nano/micro are fiber mat more induced than non-loaded PCL and chitosan-loaded PCL nano/microfiber mats for application in skin wound healing.

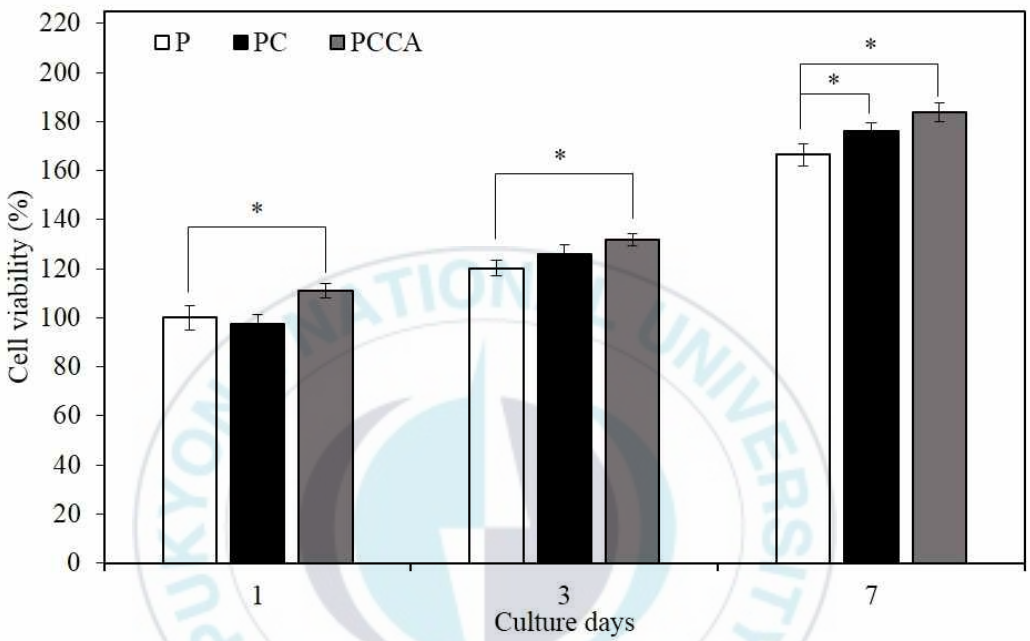

Fig. 18. NHDF-neo cell proliferation on non-loaded PCL (P), chitosan-loaded PCL (PC) and chitosan-caffeic acid loaded PCL (PCCA) nano/microfiber mats for 1, 3 and 7 days (*$P < 0.05$).

3.5. Cell morphology

The morphological appearances of NHDF-neo cells on fabricated nano/microfiber mats (non-loaded PCL, chitosan-loaded PCL and chitosan-caffeic acid loaded PCL) were obtained after 1 and 7 days of culture by SEM. Fig. 19 shows the SEM image that NHDF-neo cells attached on the nano/microfiber mats and stretched across the nano/microfiber upon proliferation. After 7 days of NHDF-neo cell culture, the number of NHDF-neo cells proliferated on chitosan-caffeic acid loaded PCL nano/microfiber mat was higher than the cell proliferated on non-loaded PCL and chitosan-loaded PCL nano/microfiber mats. In addition, indirect cytotoxicity assessment of the fabricated nano/microfiber mats indicated that the chitosan-caffeic acid loaded nano/microfiber mat was non-toxic to the NHDF-neo cells. This results show that chitosan-caffeic acid loaded PCL nano/microfiber mats were induced cell proliferation compare with non-loaded PCL and chitosan-loaded PCL nano/microfiber mats.

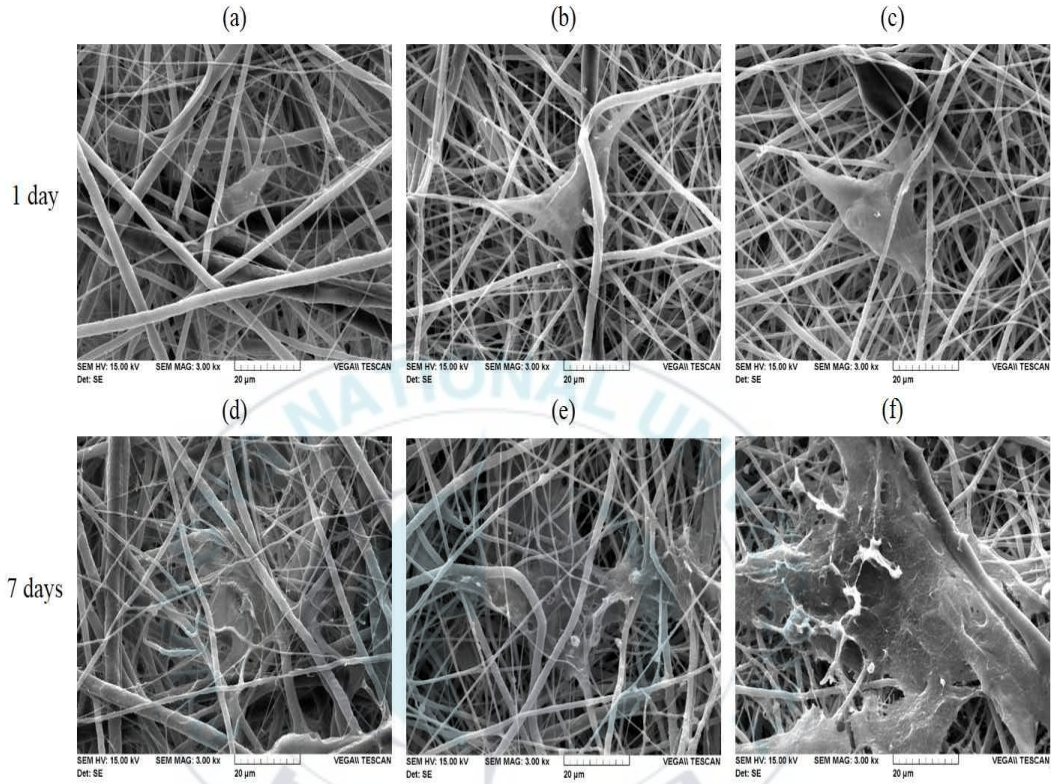

Fig. 19. SEM image of NHDF-neo cell on fabricated nano/microfiber mats for 1 (a, b, c) and 7 (d, e, f) days. Non-loaded PCL (P; a, d), chitosan-loaded PCL (PC; b, e), and chitosan-caffeic acid loaded PCL (PCCA; c, f).

3.6. Antibacterial test

The antibacterial activity of fabricated nano/microfiber mats against Gram-positive bacterium (*Staphylococcus aureus*) was tested by the viable cell-counting method. It was evaluated by counting the viable microorganisms present after incubation of fabricated nano/microfiber mats in bacteria suspension for 30, 60, 120 and 240 min. Fig. 20 and Fig. 21 showed the antimicrobial activity of fabricated nano/microfiber mats. Non-loaded PCL nano/microfiber mat did not shown antimicrobial activity. However, chitosan-loaded PCL and chitosan-caffeic acid loaded PCL nano/microfiber mats shown the higher antimicrobial activity compare with non-loaded PCL nano/microfiber mat. In particular, chitosan-caffeic acid loaded PCL nano/microfiber mat has shown high antibacterial activity against *S. aureus* compare with non-loaded PCL and chitosan-loaded PCL nano/microfiber mats.

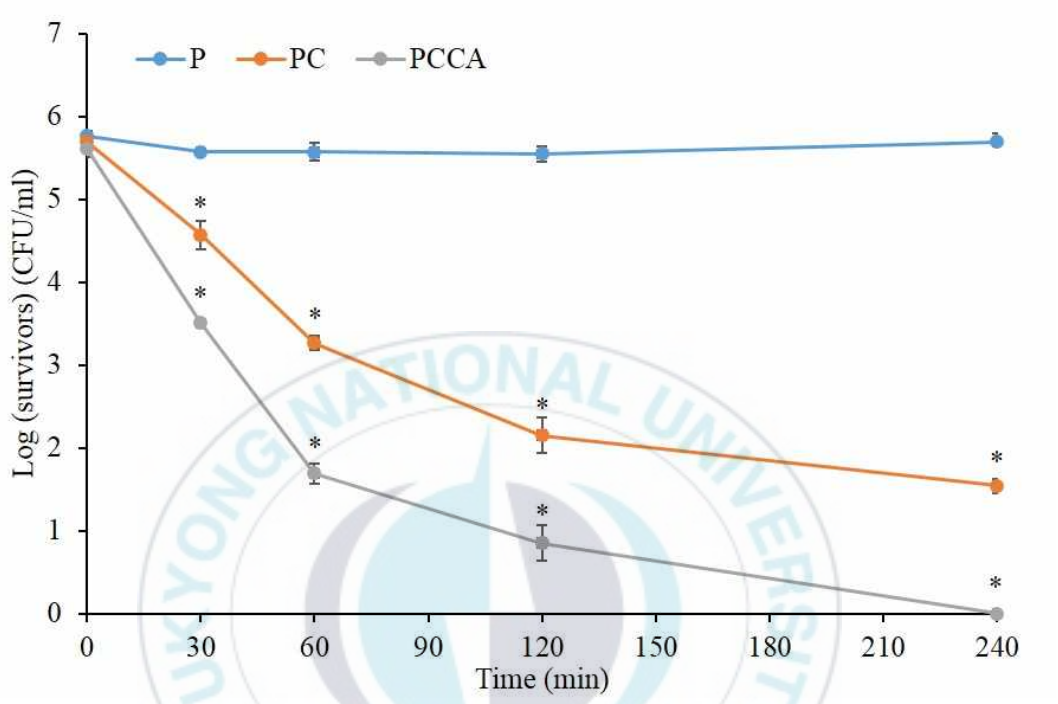

Fig. 20. Logarithmic plot of the viable bacteria cell number versus the exposure time (0, 30, 60, 120 and 240 min) for non-loaded PCL (P), chitosan-loaded PCL (PC) and chitosan-caffeic acid loaded PCL (PCCA) nano/microfiber mats against *S. aureus* (*$P < 0.05$).

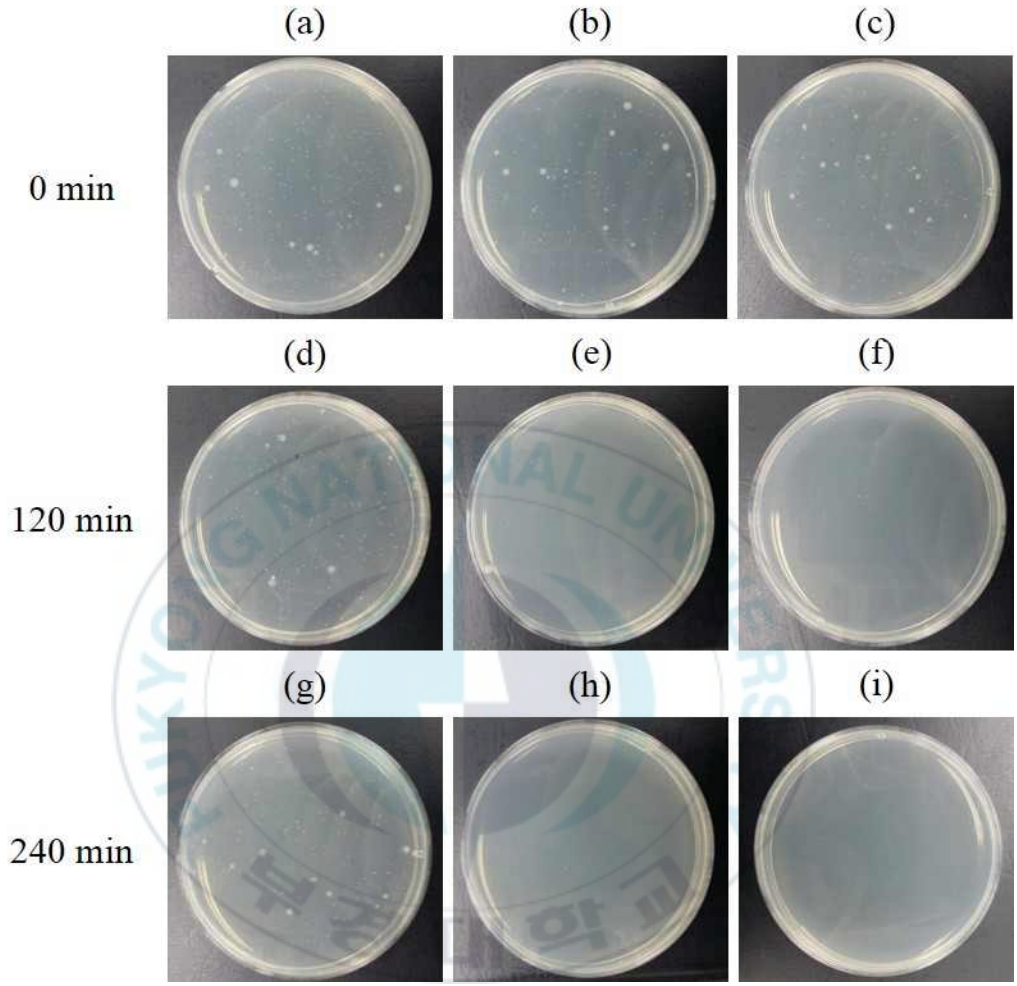

Fig. 21. Antimicrobial rate image of fabricated PCL fiber membrane against *S. aureus* at 0, 120 and 240 min. P, non-loaded PCL (a, d, g); PC, chitosan-loaded PCL (b, e, h); PCCA, chitosan-caffeic acid loaded PCL (c, f, i).

4. Discussion

Skin is the most important barrier against trespassing of external materials. However, skin can be easily injured by the various factors, and can be easily infection by microbes. After skin damage, the wound site can cause infection by microbes and cause various diseases which interrupted and failed the wound healing (Percival, Bowler & Russell, 2005). Thus, antimicrobial compound containing wound dressing is important to wound healing. Among the natural materials, chitosan have been used for wound dressing material because it has antimicrobial activity (Cheung, Ng, Wong & Chan, 2015). In addition, many studies suggested increasing of chitosan biological activity by conjugation of antioxidant compounds (as shown in Table 3). Therefore, in this present study, we fabricate the chitosan-caffeic acid loaded PCL nano/microfiber mat by electrospinning with antimicrobial activity for using wound dressing.

In the fabricating of electrospinning fiber mats, one of the major important is fabrication of non-woven fiber mats without bead formation. There are many have factors such as polymer properties, polymer solution, processing and laboratory factors which can change fiber morphology and fiber diameter (Khanlou, Ang, Talebian, Barzani, Silakhori & Fauzi, 2015). To remove bead formation of electrospinning fiber mats, we changed several parameter such as polymer solution, voltage and needle-to-collector which affect the bead formation and fiber diameter (Fig. 13 and Fig. 14). Finally, we chose the electrospinning condition which 12% PCL solution in chloroform/methanol for fabricating of non-woven fiber mats without bead formation. The non-loaded PCL, chitosan-loaded PCL and chitosan-caffeic acid PCL fiber diameter is 1.30±1.07, 1.20±1.22 and 0.94±0.68 μm (Fig.

15). This fabricated nano/microfiber size is affected the polymer concentration. Previous studies show that PCL fiber diameter was significantly increased nano to micro size with increasing of polymer concentration (Yang, Wolke & Jansen, 2008). Therefore, our fabricated nano/microfiber diameter was average 1 μm by high polymer concentration.

FT-IR spectra have been widely applied to analysis of chemical structure and bonding of materials. Therefore, to analysis structure of fabricated fiber mats, the FT-IR spectra data show that chitosan-caffeic acid loaded PCL nano/microfiber mat was successfully fabricated by increasing OH group at 3341 cm^{-1} peak. Previous study show PCL stress-strain curves that the stress-strain curve of non-woven PCL nanofibrous sample was a higher mechanical toughness and flexibility than PLA and PLA blend samples (Zahedi et al., 2012). In the present study, mechanical properties indicated that the stress-strain curves of non-loaded PCL nano/microfiber mat was similar to previous study stress-strain curves. Random and interlacing arrangement of the nano/microfibers tend to show this curves feature (Jin, Prabhakaran & Ramakrishna, 2011). In the present study, tensile modulus and ultimate tensile stress value of fabricated nano/microfiber mats is lower value than human skin tissue value. However, this is not important factor for using wound dressing materials, since the wound dressing are rarely under a high tensile strength when used at the wound site (Jin, Prabhakaran, Kai, Annamalai, Arunachalam & Ramakrishna, 2013). Therefore, electrospinning nano/microfiber mats for using wound dressing is not require a high mechanical properties.

Cell cytotoxicity is most important thing because materials for using wound dressing directly contact the wound site. Also, induction of cell proliferation by wound dressing is one of the important thing during the wound healing process that can help mediate wound

healing. In addition, cell morphology is also important factor for cell divide and cell proliferation. If cell cannot stretched or their morphology is more spherical shape, this cell cannot divide and proliferation by arresting of cell-cycle, and can undergo apoptosis (Saxena, Hwang, Huang, Eichbaum, Ingber & Orgill, 2004). Accordingly, cell cytotoxicity, cell proliferation and cell morphology on fabricated wound dressing was determined using MTT assay and SEM. The MTT assay is based on the reduction of the water-soluble yellow tetrazolium salt to water-insoluble purple formazan products through mitochondrial dehydrogenases (Ciapetti, Cenni, Pratelli & Pizzoferrato, 1993). Previous study reported that carboxymethyl-chitosan promotes cell proliferation of normal skin fibroblasts and inhibits cell proliferation of keloid fibroblasts and secretion of collagen type I (Chen, Wang, Liu & Park, 2002). Also, (Howling, Dettmar, Goddard, Hampson, Dornish & Wood, 2001) study indicated that highly deacetylated chitosan promoted fibroblast proliferation and inhibited keratinocyte proliferation compare with lower deacetylated chitosan. Other study also reported that higher diacetylated chitosan is support the cell attachment and growth compare with lower diacetylated chitosan (Prasitsilp, Jenwithisuk, Kongsuwan, Damrongchai & Watts, 2000). In addition, (Minagawa, Okamura, Shigemasa, Minami & Okamoto, 2007) study suggested that chtin/chitosan can lead to wound healing acceleration by increasing of collagenase activity and wound break strength. These several studies suggest that chitosan can induce fibroblast proliferation and wound healing. In this cell study, cell viability and cell morphology showed that chitosan-caffeic acid loaded PCL nano/microfiber mats did not show normal human dermal fibroblasts - Neonatal (NHDF-neo) cytotoxicity and induced NHDF-neo cell proliferation compare with non-loaded PCL and chitosan-loaded PCL nano/microfiber mats.

In the present study, chitosan is play an important role in antimicrobial activity. Chitosan exhibit antimicrobial activity on Gram-positive bacterial compare with Gram-negative bacterial. For these reason, chitosan have been widely applied to wound dressing with antimicrobial activity. However, chitosan have different antimicrobial mechanism between Gram-positive and Gram-negative bacteria. Many previous studies reported about antimicrobial activity of chitosan, but the actual antibacterial mechanism has not yet been fully understood. Some study (Zheng & Zhu, 2003) have indicated that water-insoluble chitosan have inhibiting growth of bacteria against *Escherichia coli* (Gram-negative bacteria) than chitoligosaccharides (COS), but other study (Qin, Li, Xiao, Liu, Zhu & Du, 2006) have indicated that chitosan decrease antimicrobial activity against *E. coli* by increasing of chitosan molecular weight compare with COS. In addition, some study reported (Strand, Vårum & Østgaard, 2003) that water-soluble chitosan with degree of deacetylation (DD) around 50% had the most effective affinity for *E. coli* and was absorbed in highest amounts, but other study reported (PARK, JE, BYUN, MOON & KIM, 2004) that 75% deacetylated chitosan showed the highest antimicrobial effects as compared with 90 and 50% deacetylated chitosan. These results suggest that antimicrobial activity of chitosan is not proportional to DD vales. Also, several studies have been focused on the cationic nature of chitosan. Gram-negative bacterial (*S. aureus*) cell walls, which more hydrophilicity and negative charge on the cell surface compare with Gram-positive (*E. coli*) cell walls, showed a stronger interaction with chitosan than Gram-positive bacteria (Qin, Li, Xiao, Liu, Zhu & Du, 2006). Lower MW chitosan can pass through the microbial cell wall (in Gram-negative bacteria) and bind with electronegative substance (such as DNA) and flocculate, while higher MW chitosan can form a polymer membrane on the microbial

surface (in Gram-positive bacteria) and prevents entering of nutrients (Zheng & Zhu, 2003). Previous study indicated that conjugated chitosan by AA-H_2O_2 redox system using caffeic acid, ferulic acid and synaptic acid have higher antimicrobial activities than unmodified chitosan (Lee, Woo, Ahn & Je, 2014). Therefore, we choose the chitosan conjugate with caffeic acid for fabricating of electorspinning fiber mats which have higher antimicrobial activity than non-conjugate chitosan. The antimicrobial test indicated that chitosan-caffeic acid loaed PCL nano/microfiber mat show higher antimicrobial activity than non-loaded and chitosan-loaded PCL nano/microfiber mats. This result suggest that chitosan-caffeic acid was applied to fabricating of electrospinning fiber mat without loss of their antimicrobial activity compare with chitosan-loaded nano/microfiber mat for using wound dressing.

5. Conclusion

In the present study, the electrospinning nano/microfiber mats was fabricated using non-loaded PCL, chitosan-loaded PCL and chitosan-caffeic acid loaded PCL solution. The characterization of fabricated nano/microfiber mats by SEM, FT-IR and UTM show that the nano/microfiber of mats was successfully formed without any bead formation. In addition, we also carried out in vitro experimentation such cell viability, morphology and antimicrobial activity. This study results suggest that chitosan-caffeic acid loaded PCL nano/microfiber mats can promote NHDF-neo cell proliferation and spreading for wound healing with higher antimicrobial activity against *S. aureus* compare with non-loaded PCL and chitosan-loaded PCL nano/microfiber mats. Therefore, this fabricated chitosan-caffeic acid loaded PCL nano/microfiber mats can be used as a biomedical materials for wound dressing and skin tissue engineering application.

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먼저 학부 때부터 많은 관심과 가르침을 주시고, 부족하지만 석사를 무사히 졸업할 수 있도록 지도와 격려로 이끌어주신 정원교 교수님께 고개 숙여 깊은 감사의 말씀을 드립니다. 또한, 이렇게 석사학위논문이 나올 수 있도록 심사와 조언을 해주신 제재영 교수님과 고석천 박사님께도 감사의 인사를 드립니다. 실험실에 들어와 여러 실험적인 지식과 여러 가지 가르침을 주셨던 천충길 교수님과 류보미 박사님께도 감사의 말씀을 드립니다. 또, 광주에서 부산까지 같이 넘어와 동고동락하며 고생을 함께한 성영이와 학위 과정 동안 실험실에 들어와 같이 실험하며 지내고 있는 현호, 민성이, 민선이, 나루, 태희, 채영이와 부족한 영어를 도와주며 같이 생활하였던 Nguyen Van-Tinh 과 Pathum chandika 및 실험실을 지나쳐갔던 많은 학생들에게도 감사의 말을 전합니다.

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