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In Vitro Cytotoxicity and Degradability Tests of Gallic Acid-loaded Cellulose Acetate Electrospun Fiber

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ABSTRACT

Electrospinning technique has been recognized as one of fabrication methods employed to produce the polymeric electrospun fibers. The utilization of electrospun fiber for active compound loading has been more recently interesting. Gallic acid is a natural phenolic compound which has several biological activities especially antityrosinase and antioxidant activities. In this research, the electrospinning was used to prepare gallic acid-loaded cellulose acetate (CA) fiber aiming for use in topical delivery system. Gallic acid-loaded CA solutions were prepared by dissolving 17%w/w CA powder and gallic acid in the amount of 2.5-10 wt% based on the weight of CA powder, respectively, in the acetone/DMAc mixture. Due to no significant affect of electrostatic voltage and deposition distance on diameter of electrospun fiber, 12 kV voltage potential and of 12.5 cm deposition distance were selected for electrospinning of gallic acid-loaded CA fibers because of economy aspect. Cytotoxicity of the prepared fibers was examined by XTT assay of cell viability. The human fibroblast cell viability of 0%, 2.5%, 5% gallic acid loaded-CA electrospun and control was 72.04 ± 5.91%, 61.84 ± 3.97%, 60.93 ± 2.28% and 100% respectively. Therefore the release of gallic acid from CA electrospun into the medium decreased the human fibroblast cell viability. To employ this device as topical products, the low amount gallic acid loaded CA electrospun should be selected. The biodegradability of prepared CA and 2.5%w/w gallic acid loaded-CA fiber was conducted in phosphate buffer pH 7.4, collagenase type I solution and lysozyme solution. The biodegradability was measured at 2, 4, 6 and 8 weeks. The results indicated the rather low biodegradability of plain CA fiber and gallic acid loaded-CA fiber in three media. SEM study of tested fibers after drying with lyophilization technique confirmed the durability of these fibers from enzymatic degradation. Therefore gallic acid loaded-cellulose acetate fiber was rather stable for enzymatic degradation. However this polymeric fiber has been claimed to be degraded with lipase like enzyme from some bacteria.

Keywords: Gallic acid, electrospun fiber, cellulose acetate, cytotoxicity, degradability.

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INTRODUCTION

Electrospun fiber has been loaded with natural active compounds since this system exhibits many amazing characteristics owing to their small size, high porosity and high surface-to-volume ratio with vast possibilities for applications [1]. The electrospun fibers are often collected as randomly oriented structure the form of non-woven mats owing to the blending instability of the highly charged jet [2]. Cellulose acetate (CA) is acetate ester of cellulose typically which is the primary structural component of cell walls of plant [3]. There are wide applications of this polymer since it can be employed in the form of fiber, membrane, or film. The utilization of this polymer for natural active compound loading has been reported [4]. It is possible and interesting for using electrospun fiber for topical or transdermal delivery of active compounds. Electrospinning technique has been one of the methods applied for fabrication of fiber. This interesting process is rather complex with the resulting fiber characteristic being influenced by numerous materials, design, and operating parameters [5]. Processing variables especially spinning voltage and collector distance were the most important affected the morphology electrospun fiber [6]. Typically, the fiber morphological and physical characteristics of prepared fiber were apparently evident depending on the applied electrical potential and deposition distance [7].

Gallic acid (3, 4, 5-trihydroxybenzoic acid) is a naturally occurring phenolic compound which has several biological activities including antioxidant, antityrosinase, antimicrobial, anti-inflammatory and anticancer activities [8-10]. Because of its bioactivity it should be useful for skin. However, the main limitation of gallic acid is its poor water solubility (11.5 mg/mL) [11, 12. Therefore the high surface to volume of fiber fabricated with electrospinning should promote the release of gallic acid into the skin.

The gallic acid loaded-CA electrospun fiber was prepared in this study. The process parameter for electrospun fiber preparation was investigated. The indirect in vitro cytotoxicity and degradability of prepared fibers were studied

MATERAILS AND METHODS

Materials

Gallic acid (G) (Sigma-Aldrich Chemic GmbH, Buchs, Spain) was used as received. Cellulose acetate (CA; Mw = 30,000 DA; acetyl content = 39.7 w%; degree of acetyl substitution 2.4) was purchased from Sigma-Aldrich (Switzerland). Acetone and N, N-dimethylacetamide (DMAc), were purchased from Labscan (Asia), Thailand. Collagenase enzyme Type I (activity 214 Unit/mg) was supplied from Bang Trading Ltd. Dulbecco's Modified Eagle's Medium; DMEM (Sigma-Aldrich Chemie, GmbH, Steinheim, Germany), fetal bovine serum; FBS (Analytical Grade, California, USA), lysozyme (activity > 69,000 Unit/mg, hen egg-white, Sigma-Aldrich, Oakville,Canada) were used as received. Primary fibroblast cells, passage 8 were used in

cytotoxicity test. XTT cell proliferation kit (Roche Diagnostics, Indianapolis, USA) was used as received. Sodium hydroxide, NaOH) (Lab-scan Co., Ltd.), potassium phosphate monobasic (Riedel-de Haen Co., Ltd.), sodium chloride (SR Lab, Bangkok, Thailand), sodium phosphate (B/No.AF405300, Ajax Fineche ltd.) were analytical grade.

Methods

Fiber preparation by electrospinning

The CA powder was dissolved in 2:1 v/v acetone/DMAc to obtain a 17% w/w CA solution. Gallic acid-loaded CA solutions were prepared by dissolving the same amount of CA powder and gallic acid in the amount of 2.5-10 wt% based on the weight of CA powder, respectively, in the acetone/DMAc mixture. Electrospinning of the as-prepared solutions was carried out by connecting the emitting electrode of positive polarity from a Gamma High-Voltage Research ES30PN/M692 high voltage DC power supply to the solutions filled in a standard 50-ml syringe, the open end of which was attached to a blunt gauge-20 stainless steel needle (OD = 1.2 mm), used as the nozzle, and the grounding electrode to a home-made rotating metal drum (OD = 12 cm) was covered with aluminum foil to used as the fiber collection device. The voltage was controlled by the high voltage power supply. Electrical potential of 15, 20 and 25 kV was applied across a fixed deposition distance of 10, 12.5 or 15 cm between the tip of the nozzle and the outer surface of the drum. The feed rate of the solutions was controlled at 0.1 mL/h by means of a Kd Scientific syringe pump. Electrospinning was carried out in room conditions.

Characterizations and testing

Prior to electrospinning, the as-prepared solutions were measured for their viscosity and conductivity using a viscometer (VISCO STAR Plus) and a conductivity meter (Oakton CON 6/TDS 6 Conductivity/TDS meters), respectively. The measurements were carried out at 25°C and average values for each solution were calculated from at least three measurements.

Morphological appearance of the fiber mats was observed by a JEOL JSM-6380LV scanning electron microscope (SEM). The fiber mats samples were sputtered with a thin layer of gold prior to SEM observation. The number of fiber per μm^2 was determined. Diameters of the individual fibers in the as-spun fiber mats were measured directly from the SEM images using a SemAphore 4.0 software (n=30).

In vitro cytotoxicity evaluation was investigated on the primary human fibroblast cells, passage 8 using XTT cell proliferation kit for determination of % cell viability. The prepared CA and gallic acid loaded-CA electrospun fibers were immersed in culture medium (DMEM + 10% FBS) in 96 well plastic plate for 12 h before changing the culture medium. Then 1×10^4 cells/well human fibroblast cells were added onto the test fibers and incubated at 37°C and

under 5%CO_2/95% air condition for 24 h. The human fibroblast cells incubated on 96 well plastic plates was used as the control group. The cell morphology was observed under the inverted microscope (Nikon, Eclipse TE 2000-S, Japan). After incubation for 24 h, the culture medium was removed and 200 µl DMEM without FBS was added and subsequently the 50 µl XTT solution was added into individual well before incubation at 37°C for 4 h before determination of the absorbance at 450 nm using microplate spectrophotometer (Spectra Count, Perkin Elmer, Massachusetts, USA) (n=3).

In vitro biodegradability test of CA and gallic acid loaded-CA electrospun were investigated in different media. The prepared fiber was weighed (W1) before placed in 24-well plates containing 2.4 mL phosphate buffer pH 7.4 (PBS)/well, collagenase type I solution (7.6 µg (28 unit)/mL PBS) or lysozyme solution (1.5 µg/mL PBS). These solutions were removed and filled with the freshly prepared media every week. The tested fibers were removed at different time intervals (2, 4, 6 and 8 weeks), washed with distilled water before drying at 50°C and kept in desiccators for 72 h. The dried fiber was weighed (W2) and calculated for %degradability as following:

$$\% \text{ degradation} = \frac{(W1-W2)}{W1} \times 100 \quad \ldots\ldots (1)$$

Morphological change of the CA and gallic acid loaded-CA fibers before and after biodegradability freeze-dried using a freeze dryer (type 77560–01, Labconco, Missouri, USA.) were characterized under a scanning electron microscope (Maxim2005, Cam scan, UK). The fiber samples were sputtered with a thin layer of gold prior to SEM observation.

RESULTS AND DISCUSSION

The conductivity of the 17%w/w CA solution was slightly decreased as the amount of gallic acid was increased whereas there was the apparently enhanced viscosity as present in Table 1. Both electrical potential and deposition distance did not significantly affect the number of fiber/μm^2 in the case of the 17%w/w CA solution electrospinning (data not shown). The similar result has been also reported previously [13], although typically the shape of the initiating drop changes with spinning conditions such as voltage, viscosity and feed rate. The electrostatic force was gradually increased with increasing the voltage. The split ability of droplet could be reinforced owing to the increased electrostatic force with thereafter the smaller fiber should be obtained [7]. Because the difference value of distance was rather small the apparent of this effect on fiber characteristic was not found. However some research work reported the slightly larger diameter of fiber as the collector distance increased [7]. But some research has reported the decrease of fiber diameter when the distance was increased due to the increased total path trajectory of the charged jet and uniform stretching of the jet [14]. Due to no significant affect of electrostatic voltage and deposition distance on diameter of

electrospun fiber, 12 kV potential and 12.5 cm deposition distance were selected for electrospinning of gallic acid-loaded CA fibers because of economy aspect.

Table 1. The conductivity and viscosity of CA solutions containing different amount of gallic acid.

Gallic acid (% w/w)	Conductivity ר (µS)	Viscosity (cP)
0	4.45	498.5
2.5	4.32	542.2
5	4.28	686.2
7.5	4.13	832.1
10	4.04	973.2

Table 2. Effect of gallic acid amount on diameter and number of fiber/**1 µm^2** of fiber prepared from 7%w/w CA solution using collector distance of 12.5 cm and voltage potential of 12.5 Kv.

Gallic acid (% w/w)	Diameter (nm)	Number of fiber (/1 µm^2)
0	458±227	15
2.5	702±145	23
5	716±138	18
7.5	940±142	12
10	1057±187	11

The larger fiber was obtained as the higher amount of gallic acid was loaded as shown in Table 2. There was the tendency of decreased number of fiber/µm^2 when gallic acid loading was increased (Table 2). The higher viscosity due to gallic acid addition promoted the larger fiber formation. The charged jet from high concentrate solution could withstand the coulombic stretching force therefore the smooth with larger fibers could be obtained [13]. Moreover, the low solvent in the charged jet of concentrate polymer solution was dried more easily which cumbered the elongation and thinning of fibers [7]. The high concentration solution exhibited high viscosity and also high surface tension, thereafter the stretching ability was reduced [6]. From SEM, the round fibers without gallic acid particle aggregated on fiber surface were obtained for 2.5 and 5% gallic acid loaded-CA fiber therefore the gallic acid was completely incorporated into CA matrix fiber (Fig. 1). The deposition of gallic acid particle on fiber surface was found in the case of the fiber loading with 7.5 and 10%w/w gallic acid as shown in Fig. 1. Therefore there was the limitation of loading of gallic acid into CA fiber which the loading capacity should be less than 5%w/w of CA.

To assess cell compatibility of CA and gallic acid loaded-CA fibers, primary fibroblast cell were cultured on the prepared fibers. Cytotoxicity of the prepared fibers was examined by XTT assay of cell viability. The morphological structure of test cell was not changed after test with CA and 2.5%w/w gallic acid loaded-CA fiber as shown in Fig. 2. However, the shrinkage of cell was dominant in the case of cells exposed to the fibers comprising higher gallic acid amount (data not shown). The human fibroblast cell viability of 0%, 2.5%, 5% gallic acid loaded-CA

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electrospun and control was 72.04 ± 5.91%, 61.84 ± 3.97%, 60.93 ± 2.28% and 100% respectively (Fig. 3). The % human fibroblast cell viability of gallic acid CA electrospun was significantly lower than that of control ($p<0.05$). The % cell viability of plain CA electrospun was higher than that of 2.5%w/w gallic acid loaded-CA electrospun, significantly ($p<0.05$). However this value of 5%w/w gallic acid loaded-CA electrospun was not significantly different from that of plain CA electrospun and CA electrospun loaded with 2.5%w/w. Nanofibrous silk-collagen scaffold significantly improved cell compatibility when compared with salt-leached silk scaffold [15]. Chitosan-hydroxybenzotriazole/polyvinyl alcohol blend nanofiber was not-toxic to human fibroblast cells [16]. Ultra-fine fiber of hexanoyl chitosan was non-toxic toward the mouse fibroblast [17]. Chitin nanofiber alone or with extracellular matrix protein could be potential candidates for the cell attachment and spreading of normal human keratinocytes and fibroblasts [18]. Composite nanofibrous of type 1 collagen, chitosan and polyethylene oxide fabricated by electrospinning exhibited no cytotoxicity toward growth of 3T3 fibroblasts [19]. To employ as the delivery device, the low amount gallic acid loaded CA electrospun should be selected.

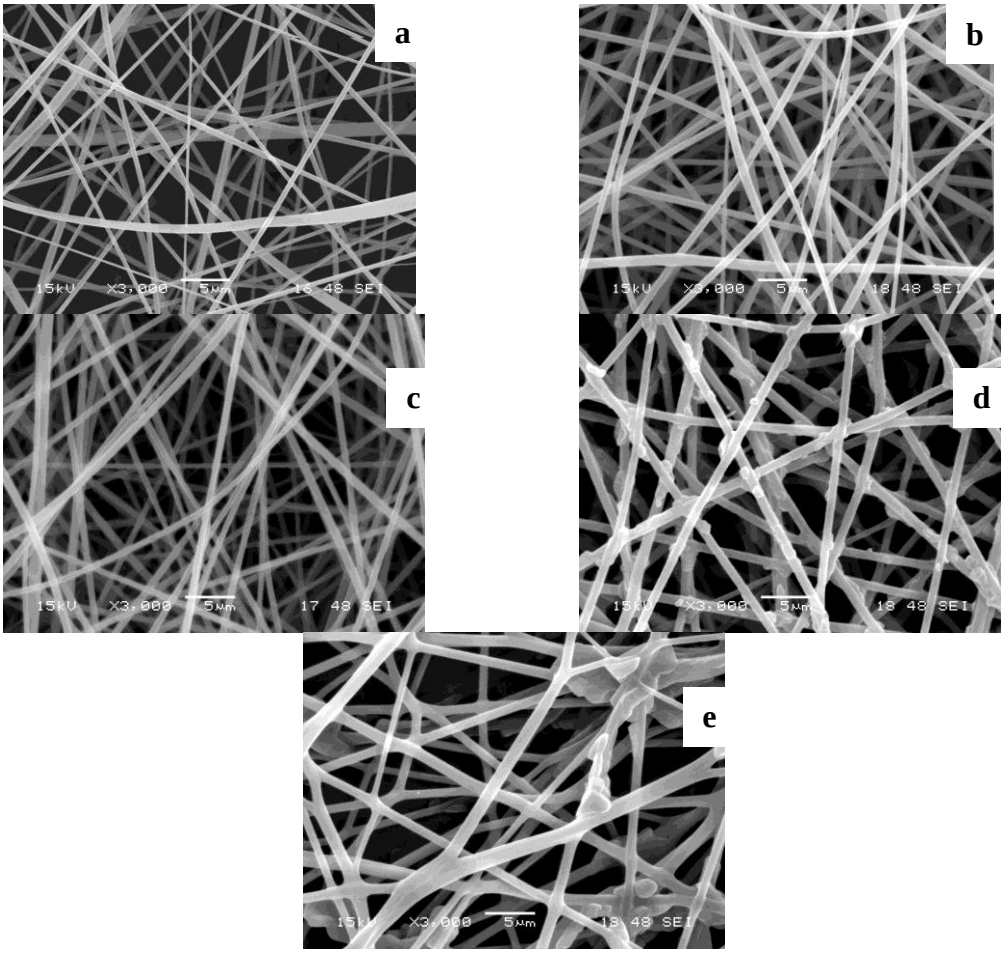

Fig 1. SEM micrograph of CA electrospun fiber prepared from the CA solution at concentration of 17 wt% with different gallic acid amount a) 0% b) 2.5% c) 5% d) 7.5% และ e) 10%w/w using the different collector distances of 12.5 cm with voltage potential of 12 kV (at magnification of 3000).

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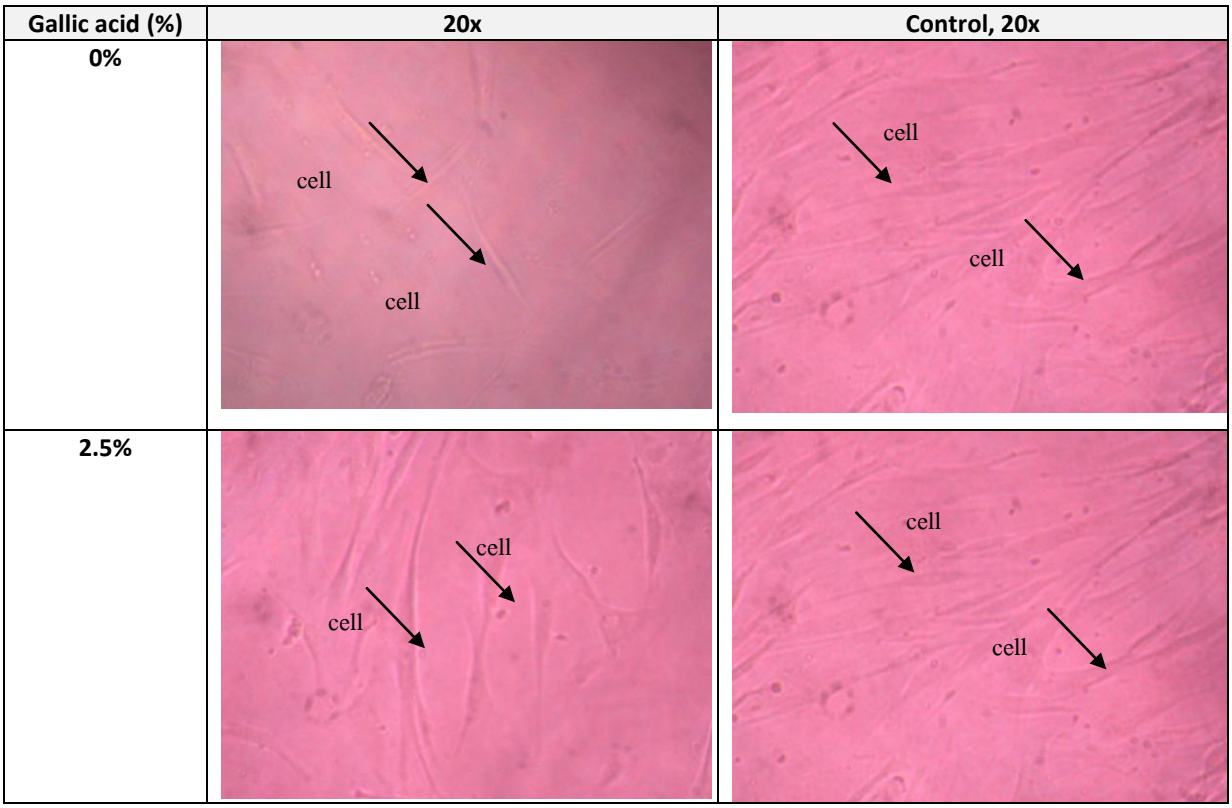

***** control = fibroblast cells seeded on plastic plate

Figure 2. Morphology of human primary fibroblast cells after incubation with 0% and 2.5% gallic acid CA fibers.

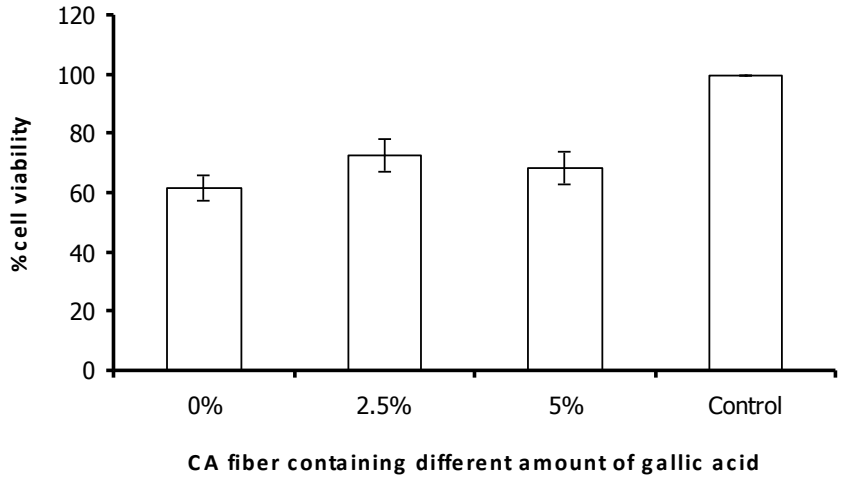

Figure 3. %Cell viability of human fibroblast cells after incubation with CA fibers containing different amount of gallic acid for 24 h using fibroblast cells on plastic well plate as control group.

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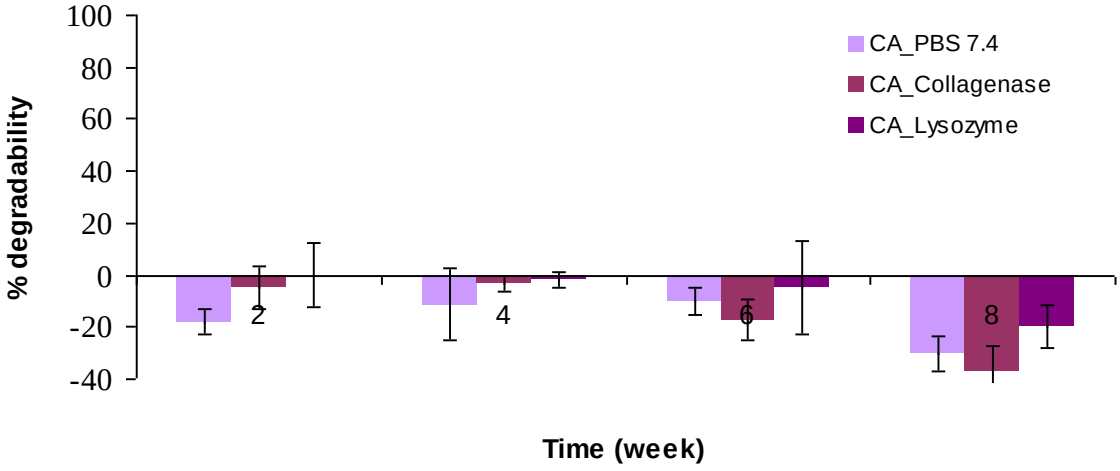

Figure 4. Biodegradability of CA fiber at different time intervals.

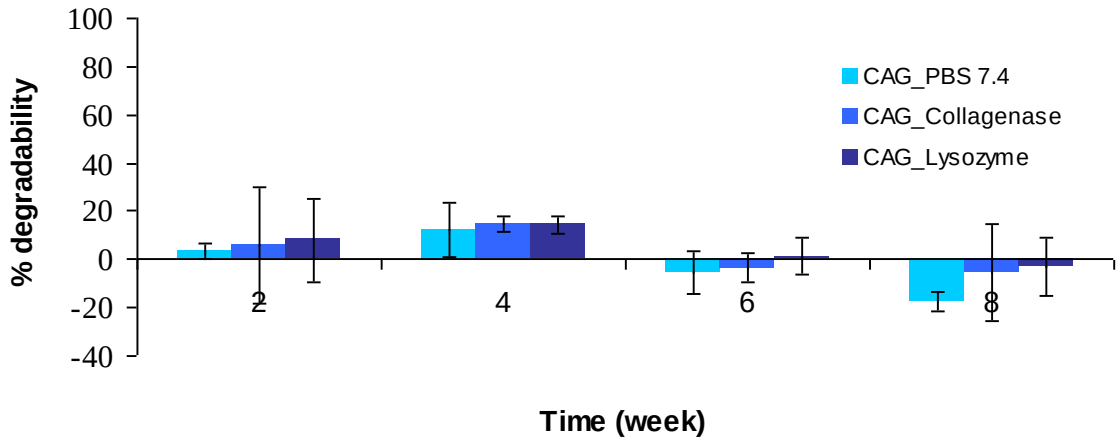

Figure 5. Biodegradability of 2.5%gallic acid loaded-CA fiber at different time intervals.

Phosphate buffer pH 7.4, collagenase type I solution and lysozyme solution were used as media for degradation test for prepared CA and 2.5%w/w gallic acid loaded-CA fibers. There was the rather low biodegradability of plain CA fiber and gallic acid loaded-CA fiber in three media as presented in Figs. 4 and 5, respectively. CA fiber could absorb the aqueous from the used media therefore the negative value was evident for each time interval for three media (Fig. 4). This may have contributed to a hydrogen formation between oxygen or hydroxyl group

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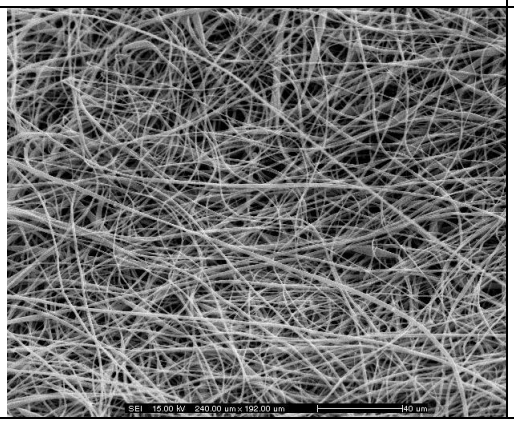
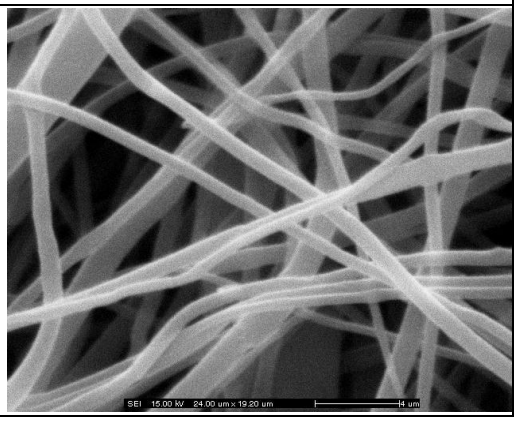
on CA structure, and oxygen or hydrogen of water molecule. Additionally, the high surface/volume or high porosity of fiber promoted the physical absorption of water with capillary action [20]. Addition of gallic acid promoted small degradation in three media at 2 and 4 weeks however the decrease of degradation was found at 6 and 8 weeks as shown in Fig. 5. The release of gallic acid at initial stage could decrease the fiber weight and thereafter the water absorption of CA fiber enhanced the fiber weight at the late stage. The SEM study of tested fibers after drying with lyophilization technique confirmed the durability of these fibers from enzymatic degradation (Figs 6-8). There was no surface erosion of fiber after short or long incubation in three media. Therefore gallic acid loaded-CA fiber was rather stable for enzymatic degradation. However this polymeric fiber has been claimed to be degraded with lipase like enzyme from some bacteria [21]. Typically, CA solubility and degradability depend on the degree of acetyl substitution (22). Highly substituted CA in CA plastic prevented the plastic from degradation and aerobic condition did not contribute much to accelerate the weight reduction of CA plastic [23] Lower-substituted CA was preferentially solubilized, leaving highly substituted CA remaining in the film. Therefore, degradation of the highly substituted fraction would be requisite for further CA film degradation [23]. Acetyl-substituted component is not uniformed distributed in CA and that preferential removal of the low-substituted fraction occurs during the biodegradation [24]. The degradability of CA by cellulase decreased with increasing the degree of substitution (DS). However some research has claimed that the combination of the deacetylating enzyme (lipase or esterase) and cellulase did not promote the degradation of CA with DS 2.4 [24, 25], therefore the structure of CA is rather difficult to broken down. The lack of biodegradability or low degradation of CA fiber signified its possibility to sustain the release of the active compounds in the future by preparing in the form of composite with other biodegradable polymers.

Figure 6. SEM micrograph of CA fiber after incubation for 2 and 8 weeks

Fiber	magnification	
	500 x	5000 x
CA 17% (2wk_PBS)		

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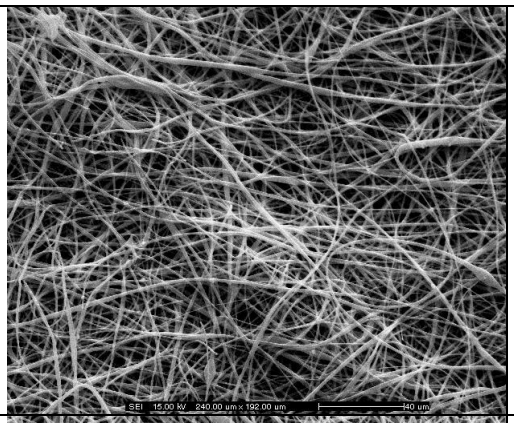
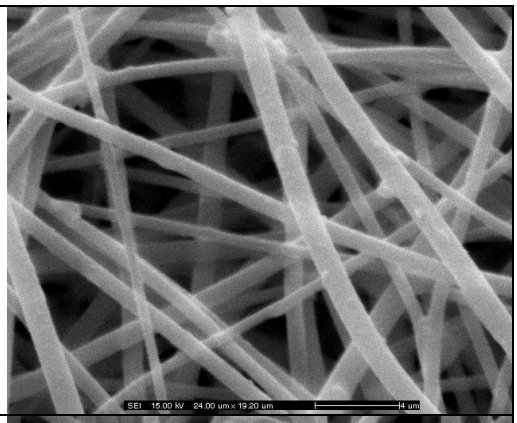
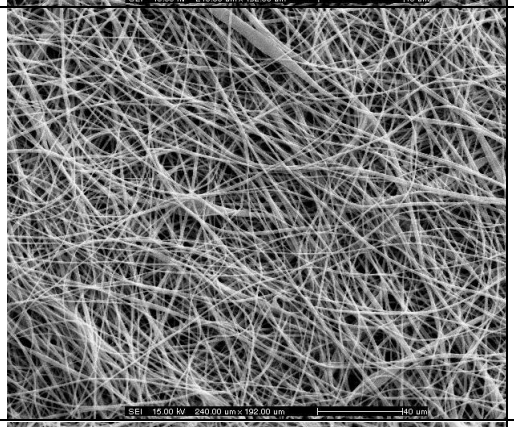
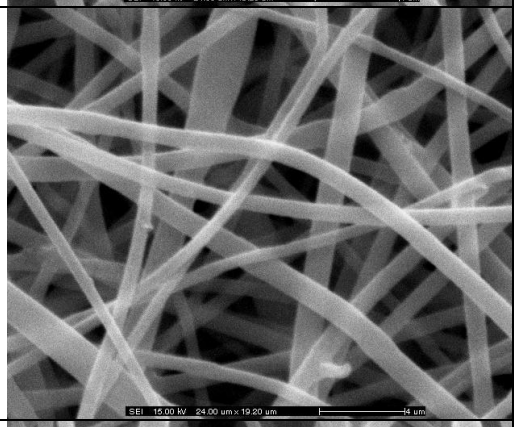
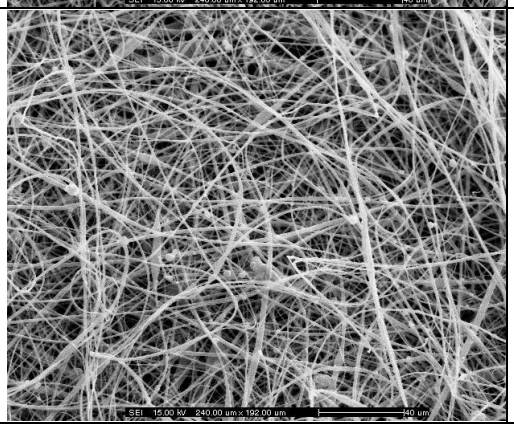
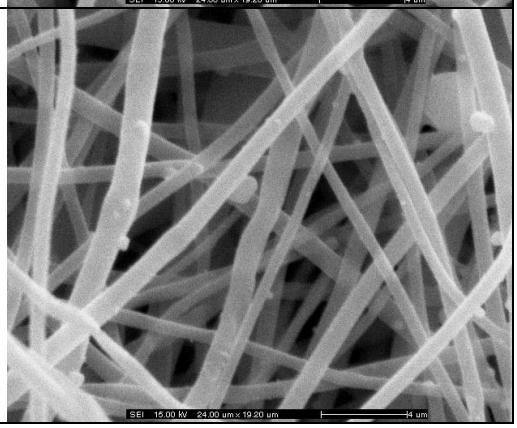
CA 17% (2wk_Lysozyme)		
CA 17% (2wk_Collagenase)		
CA 17% (8wk_PBS)		

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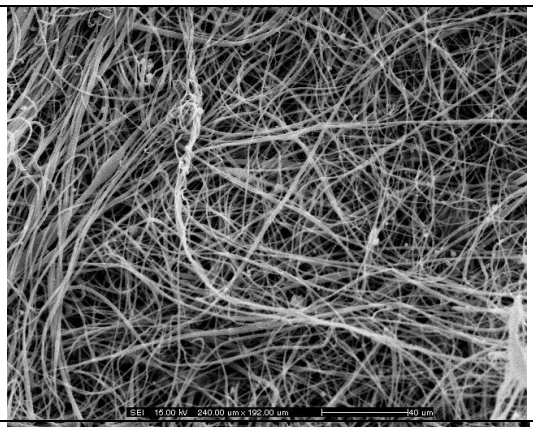
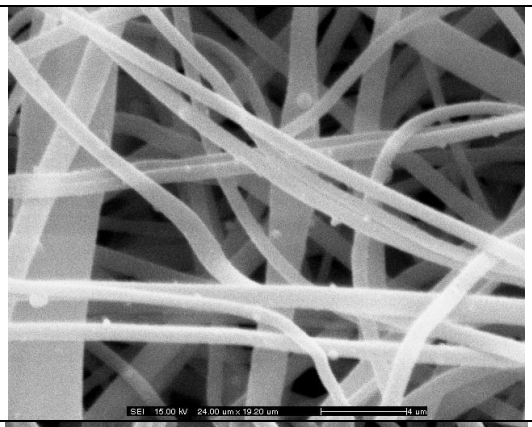
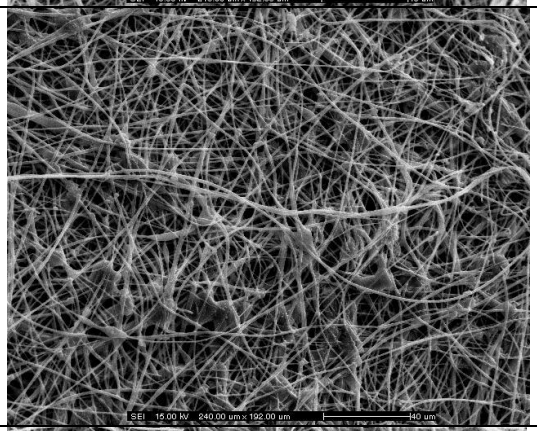
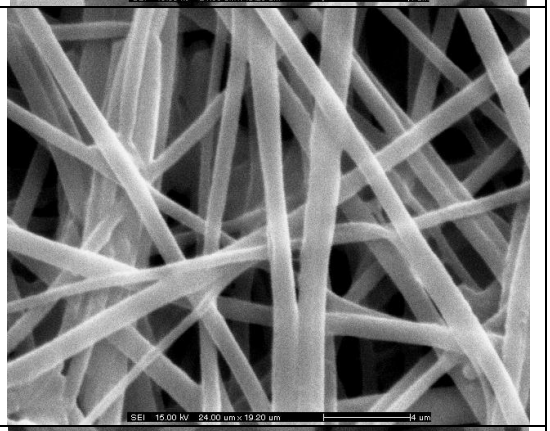
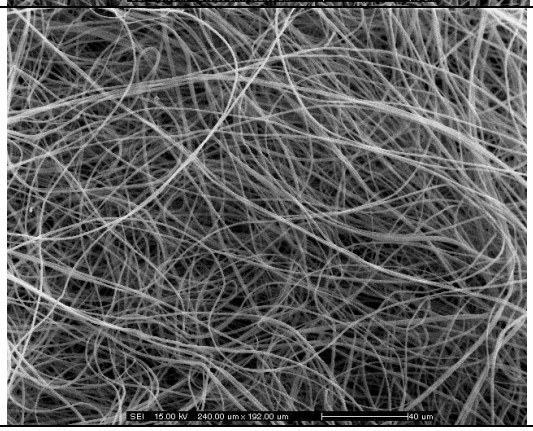
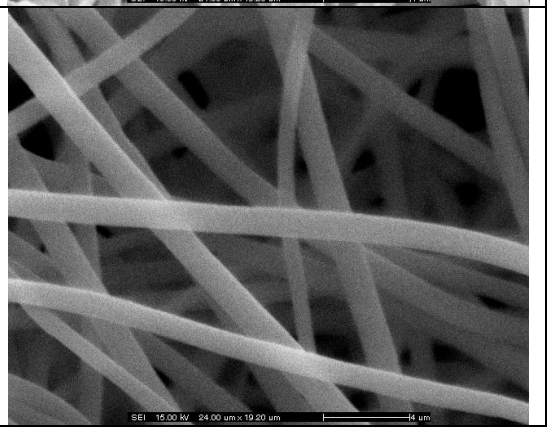
Figure 7. SEM micrograph of CA and gallic acid loaded-CA fibers after incubation for 2 and 8 weeks.

CA 17% (8wk_ Lysozyme)		
CA 17% (8wk_Collagenase)		
CA 17% + Gallic acid 2.5% (2wk_PBS)		

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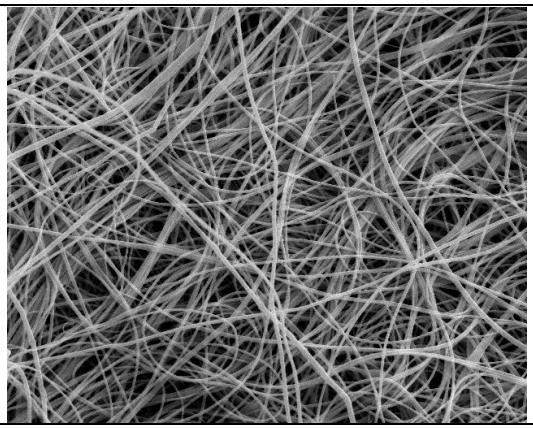
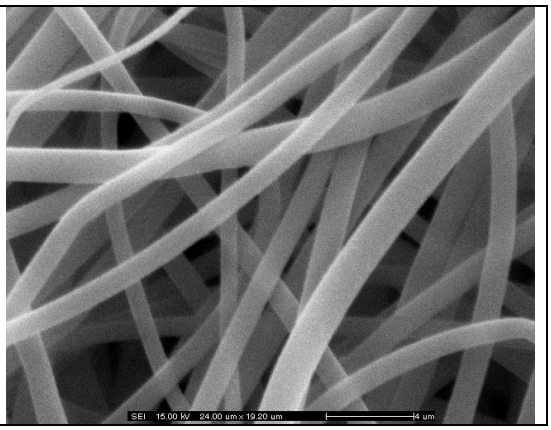
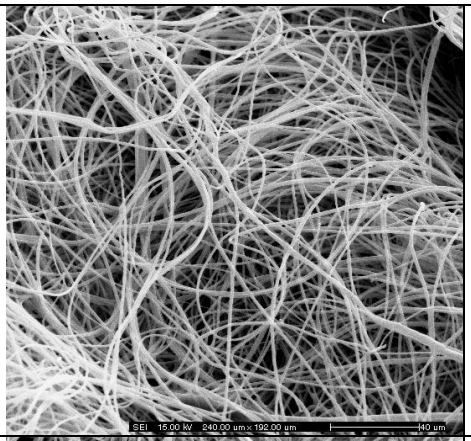
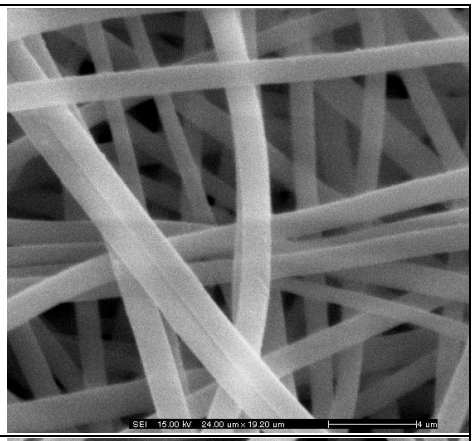
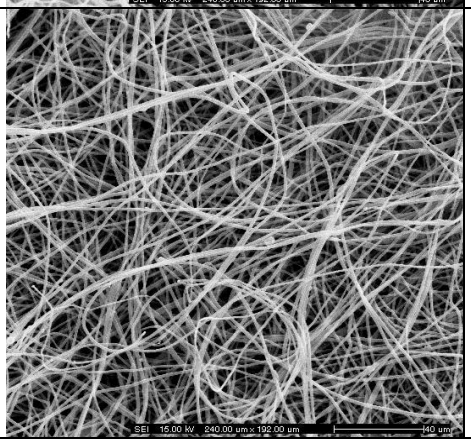
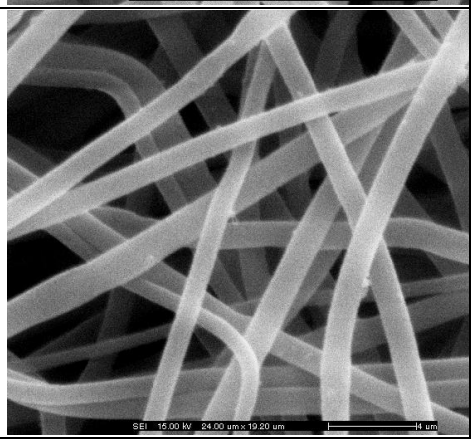
CA 17% + Gallic acid 2.5% (2wk_ Lysozyme)		

Figure 8. SEM micrograph of gallic acid loaded-CA fiber after incubation for 2 and 8 weeks.

CA 17% + Gallic acid 2.5%(2wk_Collagenase)		
CA 17% + Gallic acid 2.5% (8wk_PBS)		

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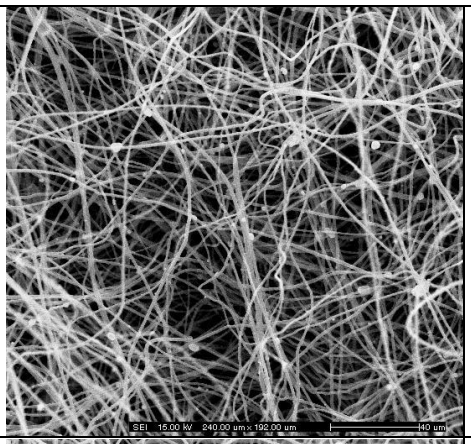
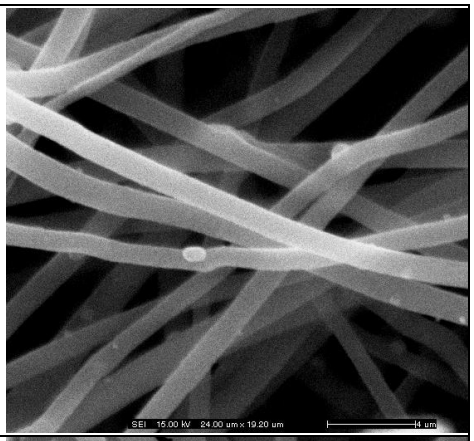
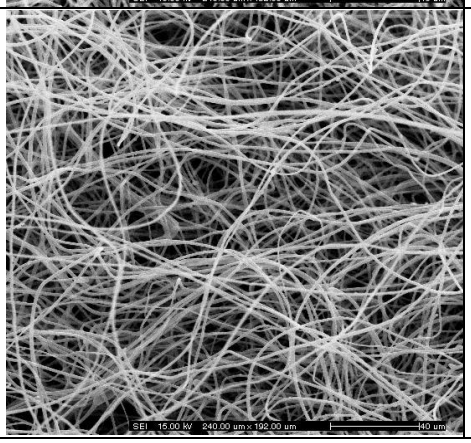
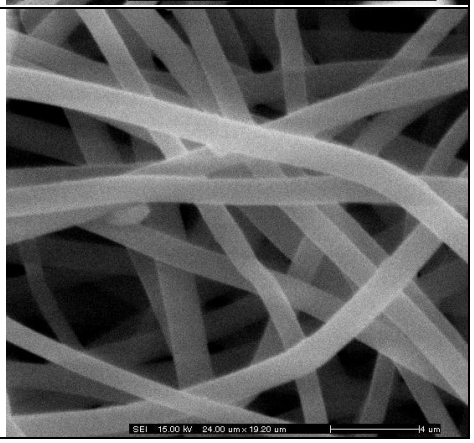
CA 17% + Gallic acid 2.5% (8wk_ Lysozyme)		
CA 17% + Gallic acid 2.5%(8wk_Collagenase)		

CONCLUSION

Gallic acid-loaded CA fiber could be fabricated with electrospinning method using acetone/DMAc mixture as solvent. Electrostatic voltage and distance did not significantly affect the diameter of electrospun fiber therefore the electrical potential of 12 kV and collector distance of 12.5 cm were employed because of economy aspect. The plain CA and gallic acid loaded-CA electrospun were rather toxic to the human fibroblast cells. The decreased human fibroblast cell viability was exhibited as the higher amount of gallic acid was incorporated into the CA electrospun fiber. Therefore the low amount gallic acid loaded-CA electrospun should be selected for using this system as the delivery device such as topical/transdermal delivery systems. Low biodegradability of plain CA fiber and gallic acid loaded-CA fibers were evident in phosphate buffer without or containing collagenase or lysozyme.

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