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Single and Composite Electrospun PCL and PLCG Fibers and Their Biological Properties

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Abstract: *Single and composite electrospun fibers derived from poly-(ε-caprolactone) (PCL) and poly-(L-lactide-co-caprolactone-co-glycolide) (PLCG) were fabricated and their biological properties were studied including cell attachment, proliferation, and cytotoxicity. The composite fibers were fabricated by two approaches: simultaneous injection of both polymers (mixed PCL-PLCG) and injection of PLCG fibers onto the layer of PCL fibers (layered PCL-PLCG). The received electrospun PCL scaffold contained complete fiber formation with the average diameter of 652.28±297.40 nm, while the electrospun PLCG, layered PCL-PLCG, and mixed PCL-PLCG had a combination of fibers, nano-sized beads, and PLCG drops. The average diameters of the electrospun PLCG, layered PCL-PLCG, and mixed PCL-PLCG fibers were 127.93 ±84.50, 399.21 ± 361.09, and 586.42 ± 418.95 nm, respectively. Among above three fibers, the average size of the mixed PCL-PLCG fibers was the greatest, however, fewer nano-sized beads and PLCG drops were formed, suggesting a mix of two polymers before reached the collector. The combining of PCL and PLCG polymers in the layered PCL-PLCG scaffold was also observed, mostly found at the interconnection of both fibers, indicating a combination of fibers after reached the collector. All fabricated fibers were non toxic to NIH 3T3 determined by MTT assay. The mixed PCL-PLCG scaffold showed the highest numbers of cells attached onto, followed by the layered PCL-PLCG and PCL scaffolds, while the PLCG scaffold showed the lowest cell attachment result. In addition, the mixed PCL-PLCG, layered PCL-PLCG, and PCL scaffolds allowed cells to proliferate with a similar number of total viable cells, whereas the PLCG scaffold showed lowest cell proliferation, which was likely because of its fast degradation in water environment.*

Keywords: *Electrospinning; Nanofibers; Composite; Cell Attachment; Cell Proliferation.*

I. INTRODUCTION

Electrospinning is an easy and effective means to produce ultrafine fibers that has been drawn tremendous interests of researchers in the past decade. This technique is involved the application of high voltages to convert a polymer solution to a fiber form. This formation occurs when the tangential stress produced by the surface charge and external

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electric field overcome the surface tension, resulting in the ejection of fluid polymer continuously toward the metal collection unit. Meanwhile, a rapid evaporation of the solvent occurs, thus leaving the submicron-size fibers or nanofibers in non-woven form on the collection device (1).

Many applications of electrospun fibers have been interested, particularly in a variety of biomedical applications, including wound healing, drug delivery, and tissue engineering scaffolds. The advantage of electrospun fibers is their unique structure of non-woven ultrafine morphology that provides high porous microstructure and interconnected pores, thus making them physically resemble the extracellular matrix of natural tissues and organs. In addition, electrospun fibers can be modified by engineering materials and adding suitable biochemical factors to restore, maintain, or improve tissue function that serve clinical tissue engineering purposes (2). Importantly, to achieve the tissue reconstruction, the fabricated material scaffolds must support cells to propagate into three-dimensional formation, including allow cell attachment and migration, deliver and retain cells and biochemical factors, enable diffusion of vital cell nutrients and expressed product, and exert certain mechanical biological influences to modify the behavior of the cell cycle. The good scaffolds, therefore, required the adequate pore size and high porosity to facilitate cell seeding and nutrient diffusion, and biodegradability to avoid a surgical removal afterward.

Many synthetic and natural polymers, therefore, have been fabricated to achieve the above requirements, including poly-(e-caprolactone) (PCL), poly(lactic acid) (PLA), poly(glycolicacid) (PGA), silk, fibrinogen, and collagen (3-8). Nevertheless, novel electrospun polymers have been continuously reported and investigated on their biological properties such as biodegradability, biocompatibility and cytotoxicity. In this paper, we have been interested in fabricating electrospun fibers derived from PCL and poly-(L-lactide-co-caprolactone-co-glycolide) (PLCG), which the latter one has never been reported elsewhere. In addition, the production of composite electrospun fibers from both polymers are also investigated. All single and composite fabricated fibers are also compared their capability to allow cell attachment and proliferation as well as their toxicity to cells.

II. MATERIALS AND METHODS

1. Materials

Poly-(ε-caprolactone), Poly-(L-lactide-co-caprolactone-co-glycolide) (70:20:10), and solvents including, 1, 1, 1, 3, 3, 3-hexafluoro-2-propanol (HFIP), methylene chloride (MC), and N,N-dimethylformamide (DMF), were analytical grades and purchased from Sigma (Sigma, USA).

2. Synthesis of Nanofiber Sheets

All electrospun fibers were fabricated using the in-house computer-controlled electrospinning system (KKU ElectroSys I, Department of Physics, Khon Kaen University). PCL solution was prepared as a 10 wt% in MC:DMF (3:1, v/v).

Electrospinning parameters of PCL nanofiber production were set at a distance of 20 cm, a voltage of 9.6 kV, and the spin rate of 0.6 ml/h. Electrospun fibers were collected on an aluminum foil sheet, and were kept at room temperature prior uses. PLCG solution was prepared as 10 wt% in HFIP and fabricated using the parameters at a distance of 20 cm, a voltage of 9.5 kV, and the spin rate of 0.5 ml/h. The composite nanofibers were produced by using 10 wt% PCL and 10 wt% PLCG solution. The layered composite nanofibers were produced by electro-injecting PCL solution as the first layer with the parameters of a distance of 20 cm, a voltage of 9.5 kV, and the spin rate of 0.5 ml/h and injecting PLCG as the second layer with the parameters of a distance of 20 cm, a voltage of 10 kV, and the spin rate of 0.6 ml/h. The mixed composite nanofibers were produced by mixing both polymers prior to electro-inject the polymers at the parameters of a distance of 17 cm, a voltage of 10 kV, and the spin rate of 0.6 ml/h.

3. Morphology Study

The morphology and diameter of the electrospun fibers were observed and determined with the use of a scanning electron microscope (SEM) (LEO SEM1450VP, UK.). The fabricated nanofibers of PCL, PLCG, layered PCL-PLCG, and mixed PCL-PLCG were cut to a piece of 1x1 cm. All nanofiber pieces were trapped with stubs and were coated with gold by sputter coater for 3 min. For study cell morphology on fabricated membranes, cells were incubated with each membrane for 24 h and then washed twice with PBS to remove non-adherent cells. Cell fixation was performed using 4% paraformaldehyde for 1 h, washed with distilled water, and washed with ethanol from 50% to 100%. After samples were dried, they were trapped with stubs and coated with gold by sputter coater. The SEM was then used to observe the samples.

4. Cell Attachment, Proliferation, and Toxicity

NIH 3T3 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C under a humidified atmosphere with 5% CO_2. When cells reached 80-90% confluence, they were trypsinized and washed with medium before counted with a hemocytometer. Each nanofiber membrane attached on aluminum foil was cut to a circle with diameter of 3 mm and sterilized by washing several times with 75% ethanol, sterilized PBS, and air-dried in a sterile lamina flow cabinet. Fabricated membranes were deposited onto each well of a 96 well plate that uncoated adhesion protein to avoid attachment of cells onto the well. Membranes were pre-incubated with MDEM+10% FBS medium for 30 min before $2.5x10^4$ cells in MDEM+10% FBS medium were added into each well. Negative control wells were also set by adding cells into empty wells and wells containing aluminum sheet. Cells were allowed to adhere to the fiber membrane for 24 h at 37°C under a humidified atmosphere with 5% CO_2. Non-adhered cells were washed out three times with PBS, then relative viable adherent cells were determined using MTT assay (Sigma, USA). Cells were incubated with 1 mM MTT solution in PBS for 4 h at 37°C under a humidified atmosphere with 5% CO_2 and after that MTT solution was removed. Formazan, the product of metabolically active cells reacted with atetrazolium salt in

MTT agent, was resuspended in 50 ml of DMSO and transferred to a new plate to measure the absorbance at a wavelength of 550 nm. Each condition was done in quanticate and each experiment was done in duplicate.

For cell proliferation assay, NIH 3T3 cells and fabricated membranes were prepared as mentioned previously. Cells of 2.5×10^4 in DMEM+10% FBS were incubated with the sterile membranes for 1, 2, 3, 5, 7, 9, and 11 days at 37°C under a humidified atmosphere with 5% CO_2. After medium was removed, viable cells in each well at each time point were measured by MTT agent as previously described. Each condition was done in quanticate and each experiment was done in duplicate.

For cell toxicity assay, NIH 3T3 cells and fabricated membranes were also prepared as mentioned previously. The membranes were incubated with 2.5×10^4 cells in DMEM+10% FBS for 24 h at 37°C under a humidified atmosphere with 5% CO_2. Viable cells in each condition were measured by MTT assay as described previously. Each condition was done in quanticate and each experiment was done in duplicate.

5. Statistical Analysis

The data were analyzed by the two-way ANOVA test. A p value of ≤ 0.05 was considered statistically significant.

III. RESULTS AND DISCUSSION

1. Fabrication of Single and Composite Fibers

Several reports have been done on the fabrication of electrospun poly-(e-caprolactone) (PCL), whereas, up to our knowledge none has been done on poly-(L-lactide-co-caprolactone-co-glycolide) (PLCG) (9-12). Thus, in this article, we are interested in fabricating both polymer as a single or composite fibers and also compared their biological properties of cell attachment, proliferation, and toxicity using *in vitro* assays. Four fiber types were electrospun by ejecting single polymer (PCL or PLCG), both polymers simultaneously (referred to as "mixed PCL-PLCG composite"), and PCL layer followed by PLCG layer (referred to as "layered PCL-PLCG composite"). Morphology of each fiber scaffold was observed under the scanning electron microscope (SEM). It was found that the electrospun PCL sample was completely formed fibers, whereas, the electrospun PLCG gave a combination of fibers, small-sized beads on fiber string, and aggregated drops (Fig 1). The bead formations were probably due to the relatively low rates of solvent evaporation and small deposition distance. For droplet formation, it could be a result of the low concentration of polymer in the solution (13). Since beads and drops were formed by PLCG solution, unsurprisingly, the combination of beads and drops of PLCG was observed along with the fibers in the sheets of layered PCL-PLCG and mixed PCL-PLCG. In addition, the flat fibrous structure was observed in layered and mixed PCL-PLCG samples. These could be explained from wet fibers that not evaporated completely before reaching the collector, therefore fibers could be flatten upon impact on the collector and undergone re-dissolution and coalescence, resulting a observation of larger fibers (14). The diameters of fabricated PCL, PLCG, layered PCL-PLCG, and

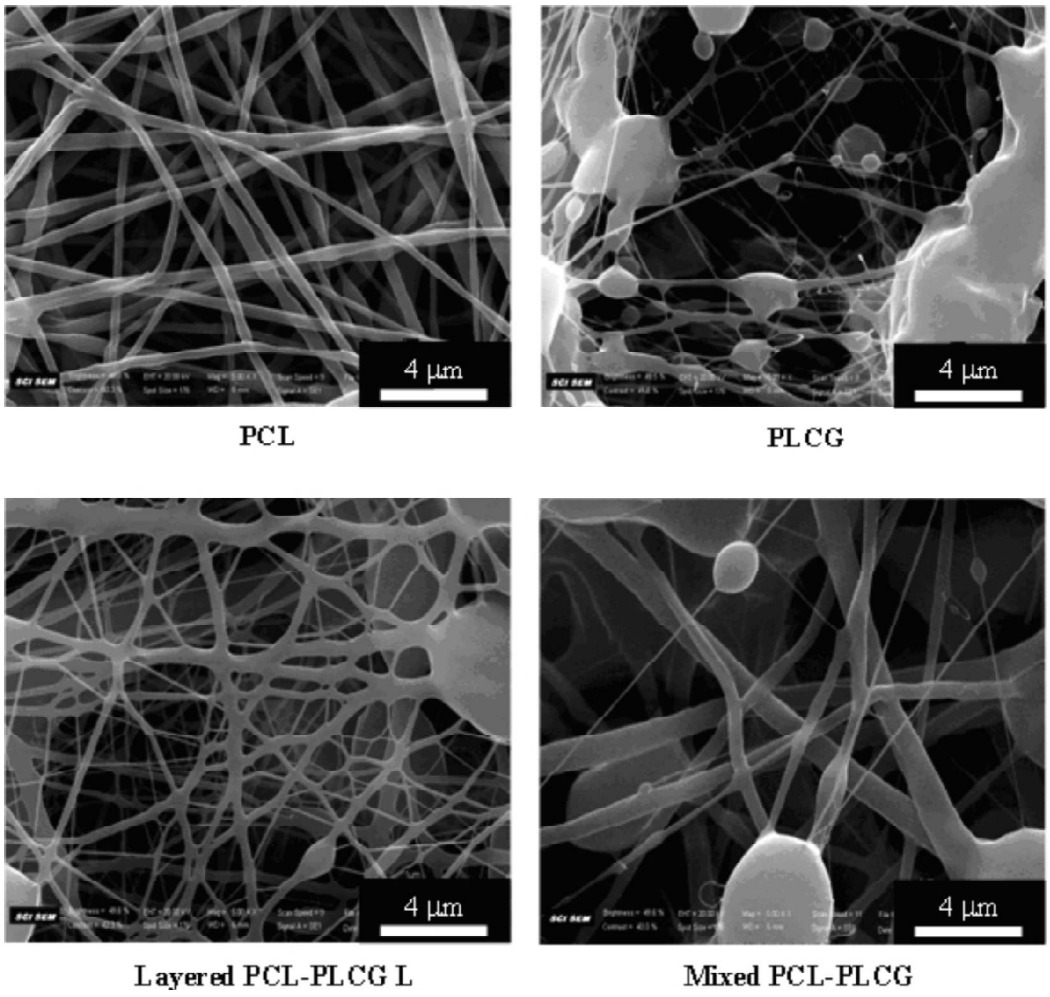

Fig. 1: Morphology of fabricated PCL, PLCG, layered PCL-PLCG, and mixed PCL-PLCG fibers. The fiber morphology was observed under the scanning electron microscope at 5,000 x.

mixed PCL-PLCG fibers ranged from 200-1400 nm, 20-400 nm, 70-2200 nm, and 100-2000 nm, respectively (Fig 2). In addition, the average sizes of the above fibers were 652.28 ± 297.40, 127.94 ± 84.50, 399.21 ± 361.09, and 586.42 ± 418.95 nm, respectively. The sheets of elecetrospun PLCG, layered PCL-PLCG, and mixed PCL-PLCG contained beads at the density of 19.94 x 10^3, 3.56 x 10^3, and 2.02 x 10^3 beads/mm^3, respectively (Table 1). In addition, elecetrospun sheets of PLCG, layered PCL-PLCG, and mixed PCL-PLCG contained polymer drop areas of 33.82%, 41.08%, and 18.53%, respectively. All above data were correlated well with the fiber morphology observed under the SEM. The electrospun PCL fibers were observed with a larger size than PLCG fibers. Layered

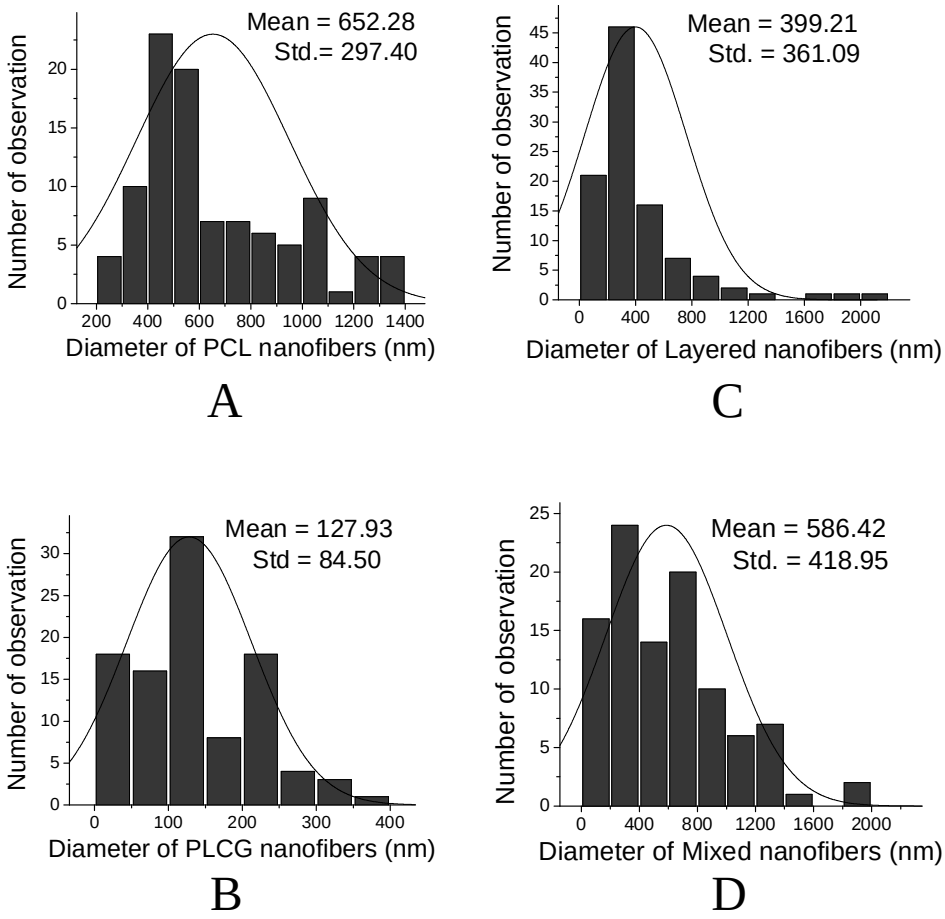

Fig. 2: The Distribution of Fiber Sizes. (A) Fabricated PCL fibers. (B) Fabricated PLCG fibers. (C) Fabricated layered PCL-PLCG fibers. (D) Fabricated mixed PCL-PLCG fibers. The diameter of fibers were measured from the random 100 selected areas of the SEM pictures.

PCL-PLCG samples contained a wide distribution of fiber sizes covered 70-2200 nm that most small size fibers were from PLCG, larger size fibers were from PCL, and the largest size fibers were from mixed polymers. The networks of fibers were also observed, which were probably formed when wet fibers of PCL and PLCG were re-dissolved at the interconnection points after reached the collector. The average fiber size of mixed PCL-PLCG sample was larger than that of layered PCL-PLCG sample. The enlarged sizes of fibers were probably caused by the mixing of wet fibers of PCL and PLCG on air, instead of the mixing on the collector that the latter one would probably give a network morphology of fibers. The unique morphology and polymer properties of each electrospun fiber were important to their biological properties, therefore, the cell attachment, proliferation, and toxicity assays were performed.

Table 1
Distribution of Polymer Beads and Drops of Fabricated Nanofiber Sheets

Nanofiber sheets	*Bead density (beads/ mm²)*	*Area of polymer drops*
PLCG	19.49 x 10^3	33.82 %
Layered PCL-PLCG	3.56 x 10^3	41.08 %
Mixed PCL-PLCG	2.02 x 10^3	18.53 %

2. Biological Properties of Electrospun Fibers

2.1. Cell Attachment

The capability of fabricated PCL, PLCG, layered PCL-PLCG, and mixed PCL-PLCG scaffolds on cell attachment were determined by allowing NIH 3T3 cells to adhere onto each scaffolds for 24 h. After removed any unbound or weak bound cells by washing several times with PBS, relative viable cell numbers were determined using MTT assay. The results showed that most cells remained attached on the mixed PCL-PLCG scaffold, followed by PCL and layered PCL-PLCG scaffolds, and PLCG scaffold (Fig 3-4), respectively. Since mixed PCL-PLCG scaffold contained network structure between PCL and PLCG from a simultaneous ejection of both polymers, it was probably the reason of having most cells remained on the more complex non-woven scaffold. From the observation, the PLCG scaffold seemed lost their integrity after incubation in the culture medium for several hours and by washing. Therefore, even though the layered PCL-PLCG scaffold contained both PCL and PLCG network, PLCG was sprayed on the top layer and easily lost their structure upon washing, thus mostly leaving only PCL fiber layer on the scaffold. Therefore, the numbers of cell attached on the PCL-PLCG scaffold were similar to the PCL scaffold after vigorously washing. The same reason could also explained why the PLCG scaffold had the less number of cells remained onto. The control wells, which are the wells with coated adhesion protein, without adhesion protein, and with sub-scaffold (aluminum foil) were also used in the experiment. It clearly showed that without coated adhesion protein, very few cells could attach onto the well. While, wells contained adhesion protein showed a higher number of adherent cells, which were similar to wells contained PLCG fiber scaffold. Unsurprisingly, the wells containing sub-scaffold also had a high number of cell attachment. This could be explained from many wrinkles on its surface that could hold cells onto. However, we believed that the number of cells adhered to each fiber scaffold were due to the fiber network, not to the sub-scaffold, since fibers were completely covered the sub-scaffold.

2.2. Cell Proliferation

Cell proliferation on each fabricated fiber scaffold was also determined. The results showed that cells were most proliferated in wells containing PCL, layered PCL-PLCG, and mixed PCL-PLCG scaffolds (Fig 5). It suggested that these scaffolds were suitable for cell adherent, therefore, they would also allow the proliferation of the cells. PLCG scaffold, which were less suitable for cells attached onto, thus, had very low number of cell proliferation result, which as low as the cell proliferation result on the sub-scaffold

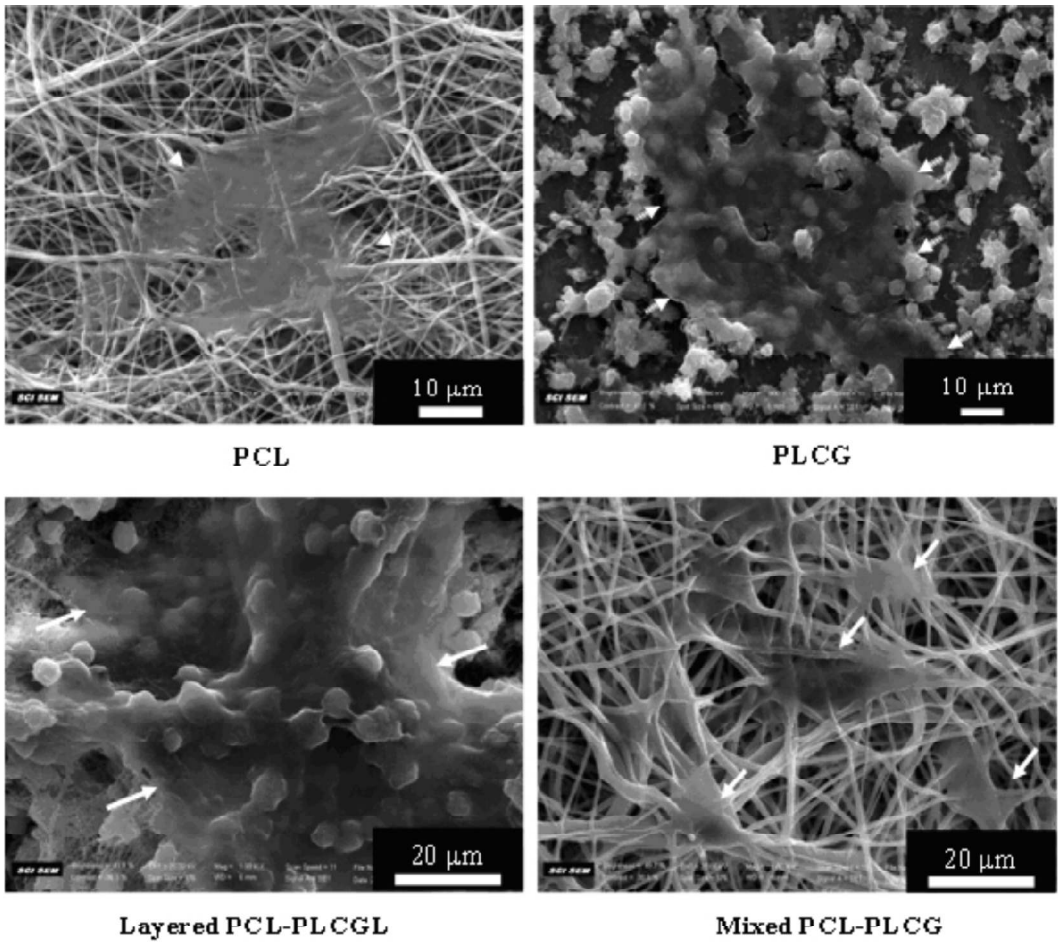

Fig. 3: SEM Pictures of Adhered cells on the Electrospun PCL, PLCG, layered PCL PLCG, and Mixed PCL-PLCG Scaffolds. Cell Locations were Indicated by white arrows. White bar scale in each picture equals 10 mm. The magnification of PCL, PLCG, layered PCL-PLCG, and mixed PCL-PLCG pictures was at 1200x, 800x, 1000x, and 1000x, respectively.

control wells. The negative control wells without adhesion protein showed the lowest number of cell proliferation. This was due to very few cells could adhere to the well as seen from the cell attachment result. It is interesting to point out that although mixed PCL-PLCG scaffold showed the best cell attachment result, the proliferation of cells in PCL, layered PCL-PLCG, and mixed PCL-PLCG scaffolds were not significantly different. This could be because of the de-conformation of PLCG fibers after the long period of incubation in culture medium. Thus, using PLCG alone as a tissue engineering materials might not be suitable due to the fast degradability. However, modified PLCG with other polymers could be an approach to slow down the degradation of the fibers in

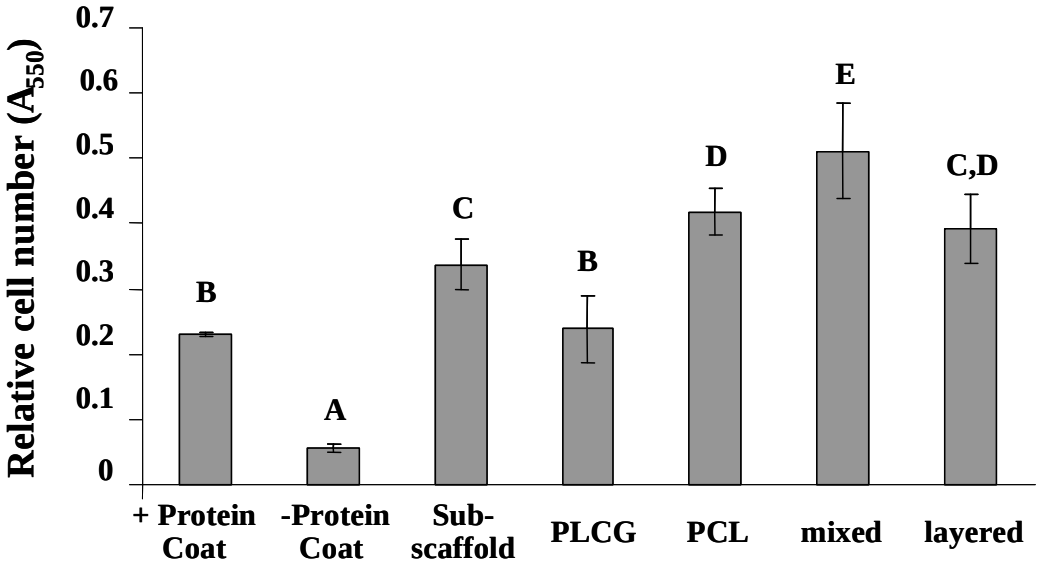

Fig. 4: Cell Attachment on Fabricated Fibers after 24 h of Incubation. Cell Remained Attachment on each Conditional well were Measured by MTT Assay. Control wells with Adhesion Protein, without Adhesion Protein, and with Sub-scaffold (Aluminum Foil) were also Included in the Experiment. The Average Data with standard deviation (n = 4) were plotted.

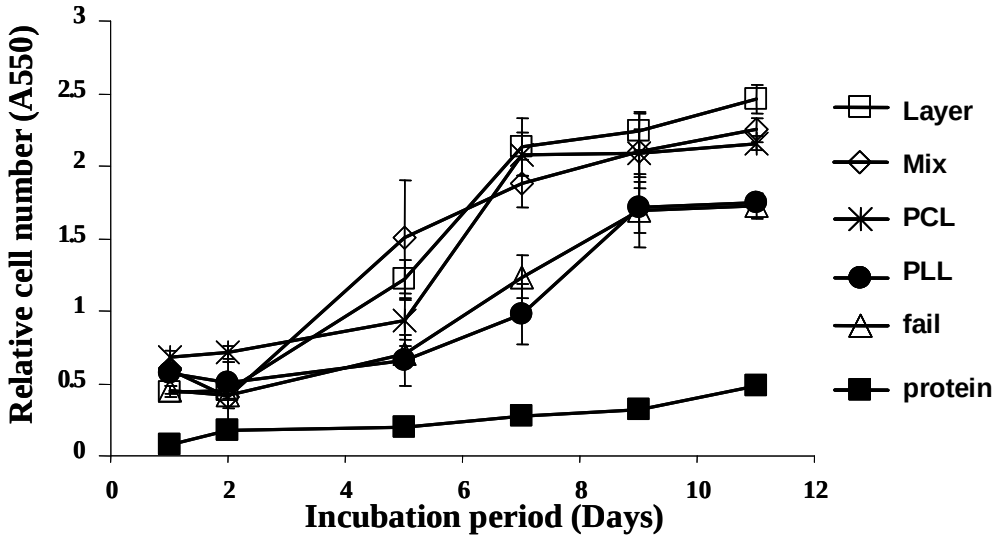

Fig. 5: Cell Proliferation on Different Fabricated Fiber Scaffolds in a Time Course of 12 Days. The Numbers of NIH 3T3 Cells on Fabricated Fiber Scaffolds at each Time Points were Determined by MTT assay. Control wells without Adhesion Protein and with Sub-scaffold (Aluminum Foil) were also Included in the Experiment. The average data with Standard Deviation (n = 4) were Plotted.

the environment and could be useful in applications that required a control release of medicinal agents of scaffolds.

2.3. Cell toxicity

In this experiment, the toxicity of fabricated fiber scaffolds were determined at 24 h after incubation with NIH 3T3 cells (Figure 6). The results showed that all fabricated fiber scaffolds were not toxic to the cells at 24 h. Control wells with sub-scaffold were also showed no toxic since there were viable cell numbers similar to positive control wells containing adhesion protein. The low numbers of cells in both controls compared to that of wells containing fabricated fiber scaffolds were a result of less cells could attach to the wells, since each wells were incubated with same cell number at the beginning of the experiment. The control wells without adhesion protein showed lowest number of viable cells, which was also because of fewer attached cells on the well surface.

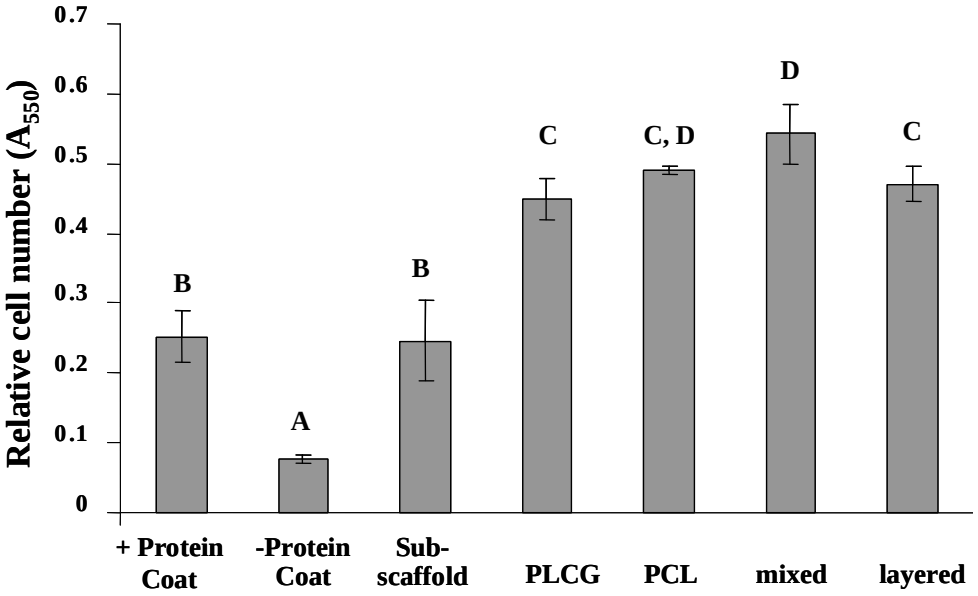

Fig. 6: Cell Toxicity in Response to Different Fabricated Fiber Scaffolds After 24 h of Incubation. Viable cell Numbers in each well were Determined using MTT Assay. Control wells with Adhesion Protein, without Adhesion Protein, and with sub-scaffold (Aluminum Foil) were also Included in the Experiment. The Average Data with Standard Deviation (n = 4) were Plotted.

IV. CONCLUSION

The present study demonstrated the production of electrospun nanofibers of single PLC and PLCG polymers, and PLC-PLCG composites, which the latter ones have never been reported elsewhere. The electrospun PLC completely formed nanofibers, while PLCG, layered PLC-PLCG, and mixed PLC-PLCG had a combinations of fibers, nano-

size beads, and PLCG drops. Unique structure and polymer composition of each electrospun fiber scaffolds resulted in different biological properties of each scaffold. Mixed PCL-PLCG scaffold had the most bound cells after several vigorous washing, followed by the PCL and layered PCL-PLCG scaffold, and PLCG scaffold. PLCG fibers seemed fastest degraded in water environment. Although, mixed PCL-PLCG scaffold allowed the most cell attachment onto, no different in cell proliferation were found in mixed PCL-PLCG, layered PCL-PLCG, and PCL scaffolds. This was hypothesized that it was because PLCG fiber network was lost after a period of incubation. In addition, all fabricated fiber scaffolds were non toxic to the NIH 3T3 cells.

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