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Biocompatibility assessments of electrospun polycaprolactone scaffolds with hyaluronic acid and/or gelatin surface modification in cultured human exfoliated deciduous cells.

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Objective: The aim of this study was to evaluate the biocompatibility of newly fabricated electrospun Polycaprolactone (PCL) scaffolds with Hyaluronic acid (HA), Gelatin (GE) or HA-GE surface modifications.
Material and Methods: The electrospun PCL scaffolds were fabricated by an electrospinning technique then they were surface modified by HA, GE and both HA and GE. Biocompatibility assessments were performed using the MTT and Scratch assays. Confocal fluorescent microscopy was used to examine cell morphology and cell distribution on the scaffolds.
Results: The MTT assays indicated that all groups had a high cell viability percentage ranging from 77.76-96.26%, except for the negative control (40%). Among the electrospun PCL scaffolds, the HA-GE-PCL group displayed a significantly lower cell viability. The migrated cell number significantly increased at 12 and 24 h in all groups. At 24 h, the HA-GE-PCL and Commercial PCL (COM-PCL) groups demonstrated higher cell migration. The confocal fluorescent microscopy illustrated that the cells distributed across the electrospun PCL fibers, whereas the cells accumulated along the edge of the mesh in the COM- PCL group.
Conclusion: In general, surface modification with HA, GE, or HA-GE on electrospun PCL scaffolds demonstrated high biocompatibility and promoted cell adhesion to the mesh surface.

Keywords: biocompatibility, gelatin, hyaluronic acid, polycaprolactone scaffold, surface modification

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Introduction

Regenerative medicine is an approach to replace/regenerate cells, tissues, or organs to restore structure and normal function [1,2]. Cleft lip and palate is one of the most common congenital defects involving the face and jaws in Thailand with an incidence of 1.40: 1000 [3]. Bone grafting is a gold standard of surgical procedures in cleft lip and palate treatment. Bone autologous graft is the safest and most effective method since it utilizes patient's own bone and provides a natural substrate for new cells to grow into the graft, to be replaced by remodeling new bone. However, it provides another surgical donor site (typically the iliac crest), with often additional morbidity pain and infections and is particularly limited volume. Allograft bone coming from tissue banks may transfer disease or lead to immunological rejection. Because of both autograft and allograft have drawbacks, scientists have long been discovering for materials that could be used to replace the transplanted bone but most synthetic bone substitutes available not yet has all benefits of autologous bone. From view of the limitations and

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owing to the increasing number of bone grafting procedures nowadays, surgeons are looking for advanced therapies [4,5]. Aside from surgical repair of the defect, the use of regenerative procedures may result in an optimal outcome [6].

The concept of regeneration involves three components: cells, scaffolds, and biologic molecules [1]. Types of scaffold play a crucial role in final material property and morphology. A polycaprolactone (PCL) scaffold is a synthetic scaffold that slowly degrades under physiological conditions whose degradation products do not change the local pH and is non-toxic. PCL has also been widely used to fabricate porous 3-dimensional (D) scaffolds for tissue engineering research. The manufacturer has stated that the pores of these products are 100% open and interconnected, which make it easy for cells to be seeded throughout the porous scaffold and for nutrients and cell metabolic waste to be exchanged. However, one disadvantage of PCL is that it is hydrophobic, resulting in poor wettability and does not allow for cell attachment. Surface modification is a technique to generate polymers that are more hydrophilic and biocompatible [7]. Disadvantages of electrospinning were also provided as it was difficult to make a large volume of scaffold at a time and time- consuming.

Scaffold design is another factor influencing cell attachment and proliferation. An appropriate scaffold should have a 3D porous structure, which allows cells from the surrounding tissue to be recruited onto the scaffold, and homogeneously colonize the scaffold to regenerate the damaged tissue. Electrospinning has been used as an effective method to fabricate scaffolds that are comprised of a network of interconnected fibers and pores. The high surface area of the electrospun mesh increases cell attachment, and the fibrous porosities facilitate nutrient and waste exchange. Moreover, the electrospinning fabrication method is simple and inexpensive. There are three rationales for improvement scaffold property according to specific characteristics related to the biological aspect, structure, and chemical composition. Common methods to modify or improve scaffold property including designing complex 3D structure with mechanical functions associated with mass transport characteristics, combining materials composition to improve structural characteristics and surface modification to improve scaffold surface properties [8, 9].

Surface coating is the simplest surface modification. The purpose of surface modification is to maintain the main material properties, while modifying the surface to improve biocompatibility via cell adhesion. Therefore, the crucial effect of a surface modification would be facilitating cell adhesion and proliferation on scaffolds [7]. Gelatin (GE) is a biopolymer from the chemical and structural degradation of collagen. Gelatin as a biopolymer that is hydrophilic, which differs from synthetic polymers. Gelatin has many biological functional groups that are suitable for surface modification in materials that are not cell-interactive. It was shown that surface-coating Polyimide with GE promoted cell attachment [10]. Hyaluronic acid (HA) is widely distributed throughout connective and epithelial tissues and is a chief component of the extracellular matrix. HA contributes to cell proliferation. This molecule participates in many crucial functions in vivo, including joint lubrication, tissue hydration, and wound healing [11]. HA also has special advantages such as being easy to produce and modify, hydrophilic, and easily degrades.

Stem cells from human exfoliated deciduous teeth (SHED) are alternative sources of stem cells because they are easily accessible and collectible. Furthermore, non-invasive collection process has been used. They were also efficient in differentiating into a variety of cell types including osteoblasts, neural cells, odontogenic cells, and adipocytes [12]. The most significant difference between SHED

and adult dental pulp stem cells (DPSCs) is that SHED are able to induce bone formation when implanted into immunocompromised mice subcutaneously using hydroxyapatite/tricalcium phosphate as a carrier vehicle [12], while DPSCs generated a dentin/pulp-like structure [13].

Currently, the effect of specific PCL scaffold surface modifications on the biocompatibility with SHED cells is unknown. Thus, the aim of this study was to investigate the biocompatibility of electrospun PCL that had been surface modified with GE, HA or both HA and GE with SHED cells. MTT and scratch assays were used to analyze the biocompatibility of the tested materials' extract at 0, 3, 6, 12, and 24 h. Confocal fluorescent microscopy was used to examine the cell morphology and cell distribution on the tested materials at day 0, 1, 3, and 7.

Materials and methods

This study was approved by the Ethics Committee on Human Rights Related to Human Experimentation of the Faculty of Dentistry at Mahidol University (COE. No. MU-DT/PY-IRB 2014/041.2110)

SHED cell isolation

Four human deciduous teeth due for exfoliation from four different anonymous donors were extracted at the Pediatric dental clinic at the Faculty of Dentistry, Mahidol University. The teeth used in this study were caries-free, no history of trauma, and showed no sign of pulp necrosis on clinical and radiographic examination.

Each extracted tooth was immediately placed into Dulbecco's modified eagles medium (DMEM) – High glucose culture medium (Gibco, Thermo Fisher Scientific #SH30022.01) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (Gibco/Invitrogen #10437-028) and 1% (v/v) Penicillin/Streptomycin mix (10,000 IU/ml Penicillin and 10,000 µg/ml Streptomycin) (Gibco, Thermo Fisher Scientific #30-002-CI) after extraction and the SHED cells were isolated from the primary teeth within 24 h after extraction. The tooth was placed on sterile gauze in a petri dish with no medium and cutting pliers were used to split the tooth. A no.15 sterile blade and sterile tissue pliers were used to remove the pulp tissue from the broken tooth. The pulp tissue was cut into 10 pieces of 1 mm using a scalpel. Two pieces of the pulp tissue were transferred to a T-25 culture flask (5 flasks total). The pulp tissue pieces were placed approximately 5-mm apart. Eight ml cell culture media was added to each flask. Each flask was labeled with: SHED, tooth number, P0, media type, Date/Month/Year. The flasks were cultured at 37°C, in a 5% CO_2 and >90% humidified air incubator. The culture medium was changed every 2 d. The cells were passaged when approximately 80% confluent. The cells were used at passages 4-6.

Electrospun Polycaprolactone (PCL) scaffold preparation

The scaffolds were prepared in three steps. The polycaprolactone (PCL) (M.W. = 70,000–90,000 g/mol, Sigma-Aldrich, St. Louis, Missouri, USA) was dissolved in a Chloroform/Methanol solvent (3:1 v/v) (AR grade, RCI Labscan, Bangkok, Thailand) at a concentration of 17% (w/v). When the PCL pellets were completely dissolved, the solutions were placed into a glass syringe (10 ml) with 21-guage needle (0.5 mm in inner diameter) and were spun into fibers using the electrospinning technique (Voltage = 20 kV, Distance to the collector = 15 cm, Flow rate = 5 ml/h) [14]. The output voltage of the power supply was read with a multitester (YX-360TR; sp®a; Thailand) and the scaffolds were washed with DI water. The PCL nanofiber scaffold was dried in a vacuum desiccator (Labconco, Kansas City, MO, USA) at room temperature

for 24 h. The PCL polymer nanofiber scaffold was cut into 4x4 mm^2 squares. Finally, the scaffold was surface treated with either gelatin (GE), hyaluronic acid (HA), or GE and HA.

Surface modification of the PCL nanofiber polymer scaffold with GE (GE-PCL)

The scaffold samples were washed with absolute ethanol (RCI Labscan, Bangkok, Thailand) and allowed to dry. The samples were aminolysed by immersing them in 6% w/v ethylene diamine (Thermo Fisher Scientific, Massachusetts, USA) for 1 h and washed with absolute ethanol and DI water to create aminolized-PCL. The aminolized-PCL was soaked in 5% w/v Glutaraldehyde (Thermo Fisher Scientific, Massachusetts, USA) in phosphate buffered saline (PBS) pH 7.4 for 3 h. The reaction was stopped by strongly washing the samples with a 100 ml DI water, and another reaction was performed using 5 g/L GE (Gelatin from porcine skin type A, Sigma-Aldrich, St. Louis, Missouri, USA) in PBS pH 7.4 at 4°C for 24 h. The scaffold was washed with DI water again and the remaining free aldehyde was blocked using 3% w/v Glycine (AR grade, Amresco, Ohio, USA) for 1 h [15]. The nanofiber was soaked in PBS pH 7.4 for 12 h to remove all non-reactive substances.

Surface modification of the PCL nanofiber scaffold with HA (HA-PCL)

The PCL scaffold was coated with HA by soaking in a 0.1% w/v HA (M.W.= 500,000-750,000, Sigma-Aldrich, St. Louis, Missouri, USA) solution for 1 h and fiber sheet was dried in a vacuum desiccator (Labconco, Kansas City, MO, USA) at room temperature for 24 h. HA solution was prepared by dissolving dry powder in distilled water to obtain the desired concentration. The scaffolds were fixed with 1 N NaOH, and sequentially washed with ethanol and DI water [16].

Surface modification of the PCL nanofiber polymer with GE and HA (HA-GE-PCL)

The GE-PCL scaffold was coated with HA by soaking in a 0.1% w/v HA (M.W.= 500,000-750,000, Sigma-Aldrich, St. Louis, Missouri, USA) solution for 1 h and the scaffold was dried in a vacuum desiccator (Labconco, Kansas City, MO, USA) at room temperature for 24 h. HA solution was prepared by dissolving dry powder in distilled water to obtain the desired concentration. The scaffolds were fixed with 1 N NaOH, and sequentially washed with ethanol and DI water [16]. The PCL scaffolds were stored at 4 °C until use.

Biocompatibility test

The MTT and scratch assays were used to determine the biocompatibility of the tested materials' extracts in three replications. There were 8 experimental groups:

1. Positive control: Thermanox plastics coverslips (NUNCTM Naperville, IL, USA)
2. Negative control: 0.1% Zinc Diethyldithio carbamate (ZDEC) Polyurethane (PU) film (RM-A) (ZDEC: FM-A; Hatano Research Institute/Food and Drug Safety Center, 729-5 Ochiai Hadano, Kanagawa 257-8523, Japan)
3. Media Control: DMEM culture medium
4. E-PCL: Electrospun PCL with no surface modification
5. HA-PCL: Electrospun PCL with surface modification with HA
6. GE-PCL: Electrospun PCL with surface modification with GE
7. HA-GE-PCL: Electrospun PCL with surface modification with GE and HA
8. Commercial PCL scaffold (COM-PCL) (3D Biotek 3D Insert™ PCL Scaffold Z687510 Sigma-Aldrich, Missouri, USA)

The tested materials' extracts were prepared following ISO 10993-12: the electrospun PCL scaffolds and the positive control (≤ 0.5 mm thickness) were soaked in culture medium at a ratio of 6 cm^2/1ml and the negative control and COM-PCL 3D Scaffold (> 0.5 mm thickness) were soaked in DMEM culture medium at a ratio of 3 cm^2/1ml. The prepared samples were

incubated at 37°C in a 5% CO_2 atmosphere for 24 h before testing.

MTT assay

The indirect 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay measures the metabolic activity of mitochondrial enzymes. The extracts were tested following ISO 10993-5 Biological evaluation of medical devices- Part 5: Tests for *in vitro* cytotoxicity. SHED cells (1 x 10^4 cells/100µl culture media) were seeded into 96-well plates and were cultured for 24 h. The media was replaced with the respective group's test extract and incubated at 37°C, 5% CO_2 for 24 h. The extract was removed and 50 µl MTT solution was added into each well. After 2 h incubation at 37°C/5% CO_2, the MTT solution was removed and 100 µl isopropanol was added to each well. The tested well plate was rocked for 30 min. The optical density of the metabolized MTT was determined using a spectrophotometer at 570 nm (Sigma-Aldrich, Missouri, USA) and the results were normalized to the control results. A decrease in number of living cells results in a decrease in the metabolic activity in the sample. This decrease directly correlates to the amount of blue-violet formazan formed, as monitored by the optical density at 570 nm. To calculate the reduction of viability compared to the blank equation is used:

$$\text{Viability \%} = \frac{100 \times OD570e}{OD570b}$$

Where OD570e is the mean value of the measured optical density of the 100 % extracts of the test sample;

OD570b is the mean value of the measured optical density of the blanks.

The lower the Viability % value, the higher the cytotoxic potential of the test item is. If viability is reduced to less than 70 % of the blank, it has a cytotoxic potential. If viability is equivalent to or more than 70 % of the blank, it has a non-cytotoxic potential.

Scratch assay

SHED cells (1x10^5 cells/1000 µl culture medium) were loaded into each well of 24-well plates. The culture plates were incubated for 24 h in a 37°C, >90% humidified, and 5% CO_2 incubator, therefore; generating a confluent cell layer. A 200 µl pipette tip was used to make a scratch along the diameter of each well to create a 1-mm gap at the bottom of each culture well. Each well was washed with 100 µl PBS twice. Each experiment extract solution (1 ml) was placed in each well (in triplicate). After 0, 3, 6, 12, and 24 h, the wells were fixed with 25% (v/v) Methanol (M.W.=32.04,Sigma-Aldrich, Missouri, USA) for 30 min, The Methanol-fixed cells were rinsed with PBS and stained with 0.2 % (w/v) Toluidine Blue O (Sigma-Aldrich, Missouri, USA) 1 h. The gap images were captured with a Nikon Eclipse TS 100 inverted microscope with accessories and NIS Element-Basic Research software (Nikon, Tokyo, Japan). The number of SHED cells that migrated into the gaps were counted using Image J software with a direct counting in three replications [17, 18].

Confocal fluorescent microscopy

The SHED attachment and proliferation in the E-PCL, HA-PCL, GE-PC, HA-GE-PCL, and Com-PCL scaffold groups were investigated using Confocal fluorescent microscopy.

Confocal Fluorescent imaging

Scaffold samples (1x1 cm^2 square) were loaded into 24-well-cell-culture plates. The GFP SHED were produced via virus transfection using pLenti CMV GFP Puro (658-5). pLenti CMV GFP Puro (658-5) was a gift from Eric Campeau & Paul Kaufman (Addgene plasmid # 17448; http://n2t.net/addgene:17448; RRID: Addgene_17448) [19]. The GFP SHED (5000 cells in 20 µl culture medium) was seeded onto each scaffold sample. The plates were placed in a 37°C, 5% CO_2 and >90% humidified incubator. The cell culture media were changed every 2 d. The cell distribution

on the scaffolds were investigated at 0, 1, 3, and 7 d. At each time point, the samples were collected and rinsed with PBS and fixed with 10% formalin for 30 min. The formalin-fixed samples were rinsed with PBS and stained with 1 μg/ml Hoechst 33342 (Sigma-Aldrich, Missouri, USA) for 10 min. The samples were rinsed in PBS. Surface images of the scaffolds were obtained using an inverted confocal fluorescent laser scanning microscope, OLYMPUS FV1000 (Olympus corporation, Tokyo, Japan).

Statistical analysis

The data is presented using descriptive statistics as mean ± SD. Numerical variables were compared among groups using the Kruskal-Wallis test for the MTT assay and the General Linear Model (GLM) Univariate test for the cell migration test. The results were analyzed using PASW statistics 18.0 (SPSS Inc., Chicago, IL, USA).

Results

MTT assay

The MTT assay results of the groups demonstrated a high percentage of cell viability, ranging from 79-100%, except for the negative control group (40%). The HA-GE-PCL scaffold group displayed a significantly lower SHED viability compared with the media control, positive control, COM-PCL, HA-PCL, and E-PCL groups (Figure 1). Among the experimental groups, the negative control group had the significantly lowest viability.

Scratch assay

A significantly increased number of migrated cells was observed at 12 and 24 h in the experimental groups, except the negative control group (Table 1, Figure 2). At 24 h, the E-PCL, HA-PCL, and GE-PCL groups revealed lower cell migration compared with the positive control, media control, HA-GE-PCL, and COM-PCL groups (Table 2, Figure 2).

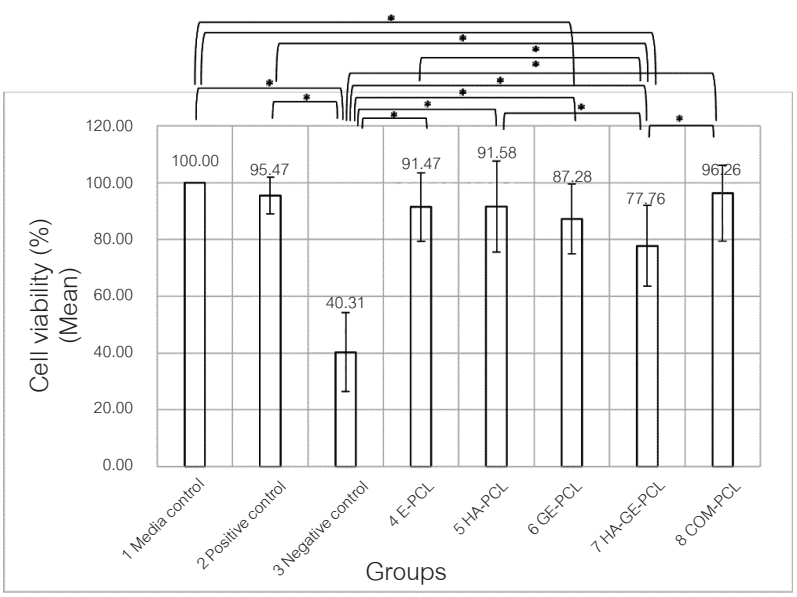

Figure 1 MTT cell viability assay results in 24 h. The Y-axis calculation of the figure showed the mean percentage of cell viability among all experimental groups.
Abbreviations: E-PCL: Electrospun Polycaprolactone scaffold, HA-PCL: Electrospun Polycaprolactone scaffold with Hyaluronic acid surface modification, GE-PCL: Electrospun Polycaprolactone scaffold with Gelatin surface modification, HA-GE-PCL: Electrospun Polycaprolactone scaffold with Hyaluronic acid -Gelatin surface modification, and COM-PCL: Commercial Polycaprolactone scaffold.
*Significant difference at $p < 0.05$, Independent-Samples Kruskal-Wallis Test

Table 1 Number of migrated cells at 3, 6, 12, 24 h

Experimental group	3 h N (SD)	6 h N (SD)	12 h N (SD)	24 h N (SD)
Positive control	0.92 (0.38)	2.06 (0.51)	38.53 (3.04)	530.42 (62.52)
Negative control	0.86 (0.30)	1.42 (0.35)	2.06 (0.37)	1.81 (1.01)
Media control	1.56 (0.57)	2.19 (0.63)	41.22 (4.21)	540.11 (45.97)
E-PCL	0.67 (0.45)	2.00 (0.89)	42.89 (4.51)	395.19 (42.79)
HA-PCL	0.58 (0.32)	1.00 (0.94)	40.97 (3.10)	374.72 (32.14)
GE-PCL	1.61 (0.51)	2.36 (0.59)	38.33 (1.76)	370.64 (31.54)
HA-GE-PCL	0.58 (0.38)	1.56 (0.48)	38.75 (2.03)	494.72 (48.90)
COM-PCL	1.36 (0.59)	2.47 (0.59)	44.17 (4.97)	540.40 (47.11)

N Number of cells, SD standard deviation

E-PCL: Electrospun Polycaprolactone scaffold,

HA-PCL: Electrospun Polycaprolactone scaffold with Hyaluronic acid surface modification,

GE-PCL: Electrospun Polycaprolactone scaffold with Gelatin surface modification,

HA-GE-PCL: Electrospun Polycaprolactone scaffold with Hyaluronic acid- Gelatin surface modification, and COM-PCL: Commercial Polycaprolactone Scaffold

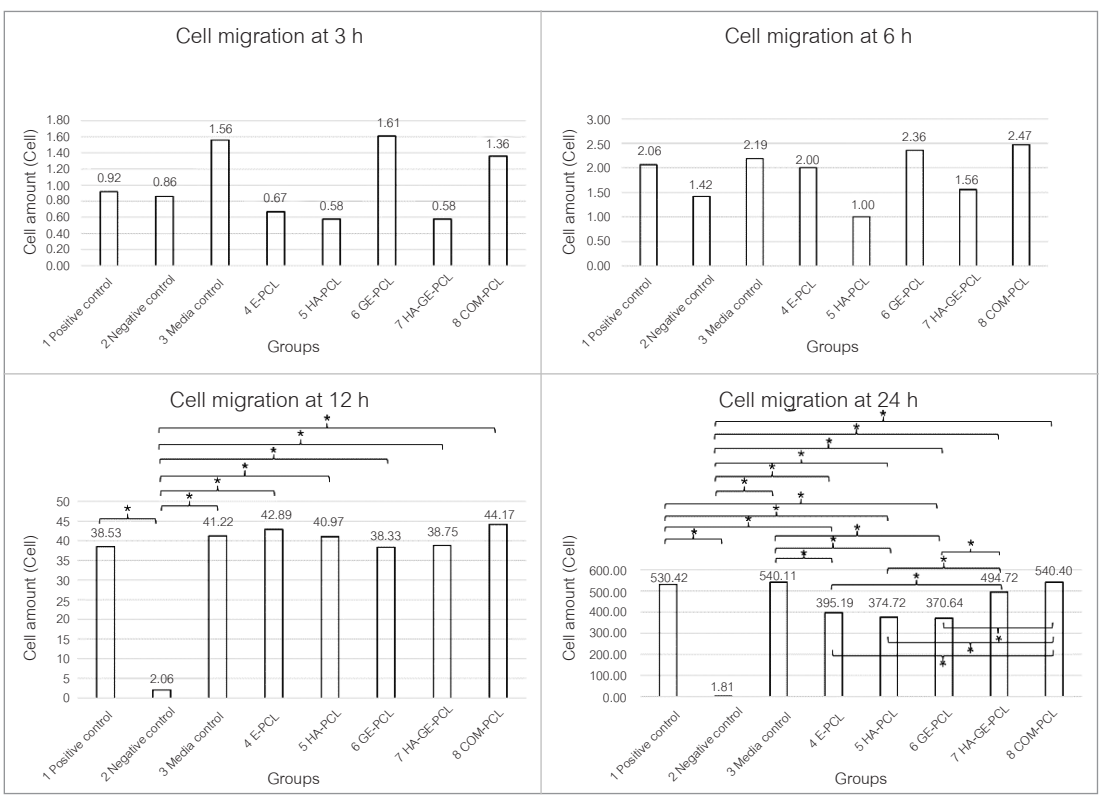

Figure 2 Bar graphs illustrated the number of migrated cells at 3, 6, 12, 24 h with statistical analysis using the General Linear Model (GML) Univariate test.

Abbreviations: E-PCL: Electrospun Polycaprolactone scaffold, HA-PCL: Electrospun Polycaprolactone scaffold with Hyaluronic acid surface modification, GE-PCL: Electrospun Polycaprolactone scaffold with Gelatin surface modification, HA-GE-PCL: Electrospun Polycaprolactone scaffold with Hyaluronic acid- Gelatin surface modification, and COM-PCL: Commercial Polycaprolactone Scaffold.

*Significant difference at $p < 0.05$, General Linear Model (GML) Univariate Test

Table 2 Statistical analysis of numbers of migrated cells at 3,6, 12,24 h using the General Linear Model (GML) Univariate Test

Experimental group	Positive control				Negative control				Media control				E-PCL				HA-PCL				GE-PCL				HA-GE-PCL				Commercial PCL			
	0	3	6	12	24	0	3	6	12	24	0	3	6	12	24	0	3	6	12	24	0	3	6	12	24	0	3	6	12	24	0	3	6	12	24
Positive control									*	*				*	*				*	*				*	*				*	*			
Negative control				*	*										*					*					*								
Media Control				*	*										*					*					*								
E-PCL				*	*				*						*										*				*				
HA-PCL				*	*				*						*										*				*				
GE-PCL				*	*				*						*										*				*				
HA-GE- PCL				*	*																				*								
COM-PCL				*	*										*					*					*								

Abbreviations:
0 = At 0 h, 3 = At 3 h, 6 = At 6 h, 12 = At 12 h, 24 = At 24 h
E-PCL: Electrospun Polycaprolactone scaffold, HA-PCL: Electrospun Polycaprolactone scaffold with Hyaluronic acid surface modification,
GE-PCL: Electrospun Polycaprolactone scaffold with Gelatin surface modification, HA-GE-PCL: Electrospun Polycaprolactone scaffold with Hyaluronic acid- Gelatin surface modification, and
COM-PCL:Commercial Polycaprolactone Scaffold
*Significant difference at $p < 0.05$, General Linear Model (GML) Univariate Test

Confocal Fluorescent imaging

The electrospun PCL scaffold groups were composed of interweaved fluorescent scaffold fibers with small porosities which was different from the COM-PCL group that had a grid-like structure (Figure 3). The viable GFP SHED cells with blue stained nuclei and green cytoplasm were observed on the scaffolds beginning at day 1. The viable cells were observed distributed over the electrospun PCL scaffold fibers, in contrast to the COM-PCL group where the cells accumulated along the edge of the scaffold mesh starting at day 3. The average diameters of E-PCL, HA-PCL, GE-PCL and HA-GE-PCL fibers were 2.5-4 μm, 2.5-5 μm, 3-6 μm and 3.5-7 μm.

Discussion

Regenerative medicine methods can optimize graft success and the healing of defective structures [6]. Scaffolds are a critical factor for successful regenerative treatment of bone defects. In the present study, MTT and scratch assays, and confocal fluorescent microscopy were performed to determine the biocompatibility of electrospun PCL scaffolds with surface modification using GE, HA, or HA-GE. To validate the validity of the assay methods, the assays were conducted using a negative control group. ISO 10993-12: 2012 recommends using 0.1% Zinc Diethyldithiocarbamate (ZDEC) Polyurethane (PU) film (RM-A) as a negative control for validating the in vitro cytotoxicity test of polymer-based biomaterials. The results of the MTT and scratch assay demonstrated that the negative control condition was toxic to the SHEDs. ISO 10993-5: 2009 indicates using a material that does not produce a cytotoxic response as the positive control. Thus, Thermanox plastic coverslips [20] that are chemically resistant and suitable for cell culture were used as the positive control in this study. A high percentage of cell viability was found in the positive control group in this study.

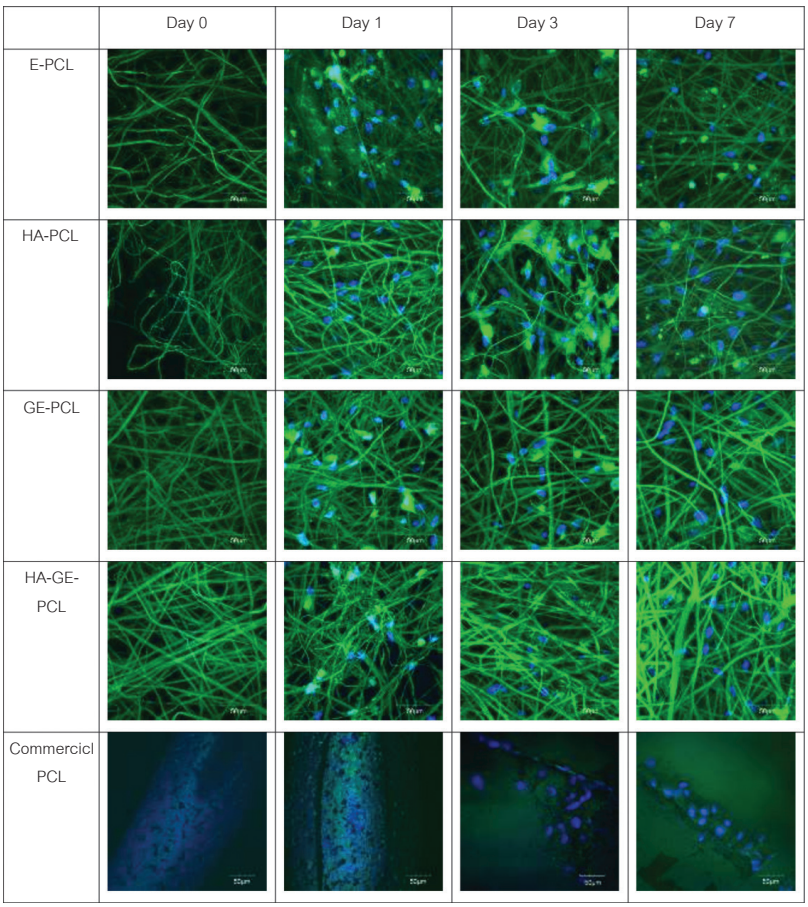

Figure 3 GFP SHED cells stained with Hoechst 33342 on the different scaffold types at Day 0, Day 1, Day 3, and Day 7. The blue-indicated oval shapes in cells displayed the blue-stained nuclei. The green-indicated fibers were the auto fluorescent of the electrospun PCL fibers themselves.
Abbreviations: E-PCL: Electrospun Polycaprolactone scaffold, HA-PCL: Electrospun Polycaprolactone scaffold with Hyaluronic acid surface modification, GE-PCL: Electrospun Polycaprolactone scaffold with Gelatin surface modification, HA-GE-PCL: Electrospun Polycaprolactone scaffold with Hyaluronic acid-Gelatin surface modification, and COM-PCL: Commercial Polycaprolactone Scaffold.

In the MTT assay, all PCL groups demonstrated a high percentage of cell viability, ranging from 77.76-96.26%. These results indicate that the electrospun PCL scaffolds with the surface modifications evaluated were highly biocompatible based on ISO 10993-5:2009. In contrast, the HA-GE-PCL scaffold group had a significantly lower cell viability compared with the media control, positive control, COM-PCL, HA-PCL, and E-PCL groups. These findings indicate that the HA-GE-PCL group had a lower biocompatibility compared with the other groups. This could be caused by their fabrication steps. The GE modification procedure used Ethylenediamine in an aminolysis reaction and Glutaraldehyde (GA) to covalently link GE to the PCL surface [21] both of which have toxic effects [22, 23]. Moreover, Sodium hydroxide (NaOH), which is toxic to human tissue, such as skin and eye tissue, was used in the HA surface coating process [24]. Among the experimental groups, the negative control group demonstrated a significantly lower viability (40%), indicating that the assay method was valid. From the MTT result, HA-GE-PCL had the lower viability compared to other scaffold groups. Nevertheless, it had the benefits from

both HA coating and GE coating. It would be valuable to try other surface modification methods or to modify the methods that were utilized by washing more after immersing in ethylene diamine in the GE surface modification and after fixing with sodium hydroxide in the HA surface modification.

The biocompatibility of the tested materials was further investigated using the scratch assay. The present study found a significantly increased number of migrated cells at 12 and 24 h in the experimental groups, except the negative control group. At 24 h, the HA-GE-PCL, COM-PCL, the positive control, and media control groups presented higher cell proliferation and migration compared with the E-PCL, HA-PCL, and GE-PCL groups. Our findings are similar to Heris et al. [25] who reported that HA-GE promoted cell proliferation and migration in a microgel-reinforced composite hydrogel scaffold. The potential of using HA in tissue regeneration was also shown in other studies evaluating cell migration by human corneal epithelial cells in vitro [26] and increased human meniscus regeneration [27]. GE promoted directional migration by endothelial cells in vitro [15]. An injectable gelatin-based hydrogel induced the migration of neuroblasts towards and along the implant tract [28]. However, interpretation of these results should be done with caution. The cells were examined after a maximum of 24 h, which is considered a short observation period. If this study was performed with a longer observation period, the differences between the groups would be more distinct.

The MTT and cell migration results were similar in the groups, however, the descending order between groups in the two assays was different. Both assays implied that the electrospun PCL with surface modifications were highly biocompatible. However, the observations of differences of the descending order of the groups between MTT and scratch assay could be caused by the mode of each interpretation. The evaluation of cell viability using an MTT assay relies on the mitochondria activity of the viable cells. The rate of color conversion from yellow to purple indicates the amount of formazan formation during the observation period reflects mitochondrial activity. However, the observed mitochondrial activity could be influenced by the amount of intracellular glucose concentration and the number of mitochondria in the cells [29]. In contrast, the scratch assay illustrates cell viability, proliferation, and migration. A study found that there are two main types of factors influencing migration activity, cell factors and environmental factors [30]. The cell factors are controlled for using only the SHED cells include the organization and dynamics of the cell cytoskeleton, cell-to-cell and cell-to-the material surface adhesion. Cell migration is also influenced by the physical environment. Cells adjust their activity and morphology to the environment, resulting in variations in cell morphology, migration mode, and migration speed.

The confocal fluorescent images illustrated that the viable GFP SHED cells with blue dyed nuclei and green cytoplasm were present on the PCL scaffolds from day 1-7. Interestingly, the viable cells were found on the mesh fiber surfaces of the electrospun PCL scaffolds, in contrast with the COM-PCL scaffolds where the cells were found only in the groove of the COM-PCL scaffold starting at day 3. These results indicate that the cell proliferation and migration on the COM-PCL scaffold is extremely limited. Thus, the COM-PCL scaffold may not be appropriate as a regenerative scaffold. Bisbenzimide Hoechst 33342 is a commonly used blue fluorescent nuclear-specific stain for the AT-region of double-stranded DNA. This dye had been used for identifying living cells based on DNA content. This stain has been used in visualizing chromatin distribution, flow cytometry for DNA determination, to demonstrate apoptotic cell death. A cell undergoing apoptosis develops membrane pores and this could be

detected by staining with Hoechst 33342 and detected using a fluorescent microscope [31]. Our results implied that the cells seeded on the PCL scaffolds had not undergone apoptosis because the well-defined nuclei of the viable cells could be observed.

The confocal fluorescent images demonstrated that the electrospun PCL scaffold groups had interweaved fluorescent scaffold fibers with small size porosities, in contrast with the COM-PCL group, which had a grid-like structure. The interweaved scaffold fibers promoted cell adhesion based on the cells observed on the electrospun PCL mesh surface beginning at day 1. Moreover, in addition to the interweaved mesh surface, the surface modification with HA and GE enhanced cell adhesion to the scaffold surface as evidenced by the distributed viable cells on the mesh fibers. These observations are similar to other studies that found that GE [32] and HA [33, 34] promoted cell adhesion and wound healing. The results from the confocal fluorescent microscopy confirmed the biocompatibility of the electrospun PCL as observed in the MTT and scratch assays.

Electrospinning is a process that can generate fiber mesh scaffolds with high porosities, large surface area-to-volume ratios, and variable fiber diameters. By modulating a combination of solution and processing variables (e.g., the polymer solution concentration, flow rate, collector distance, and applied voltage), fibers with diameters up to 10 μm can be produced. Fiber diameter can influence a variety of scaffold properties, such as porosity, pore size, and surface area. These properties are important in determining the function of scaffolds in tissue engineering applications [35]. In our study, the PCL pellets were dissolved in a Chloroform/ Methanol (3:1 v/v) because this was a common solvent used for electrospinning PCL in the literatures [14,35,36]. The PCL solution was at a concentration of 17% (w/v) as mentioned in the literatures [38,39]. Dias *et al*., 2013 studied the effect of the solvent type and concentration on the morphology and characteristics of PCL scaffolds. They discovered that better electrospun meshes were produced with the solutions containing 17 (w/v) PCL dissolved in organic solvent and the results showed for low polymer concentration no spinning is obtained. The diameter of the tip when performing electrospinning was 21-guage needle which was 0.5 mm in inner diameter. This study used the diameter size as previous studies [40]. The size of the tip also influences the size of produced fibers according to the systematic review [9]. The size of produced fibers would have the effect on biocompatibility as the fiber size would affect the porosity and pore size of the produced scaffold [41]. Therefore, the fibrous scaffolds encourage cellular adhesion and proliferation to guide a growth [42].

Heydarkhan-Hagvall *et al*. stated that there was several concerns including pore size and morphology, porosity, mechanical properties, surface properties and biodegradability, which must be considered in scaffold design. Of these, pore size and porosity are the most critical features which affect cell attachment, proliferation, migration, and/or differentiation [43]. The high porosity admits the efficient exchange of nutrients and metabolic waste between the scaffold and environment and contributes a high surface area for local and sustainable delivery of biochemical signals to the seeded cells [44]. The optimal pore size for cell attachment, proliferation, and migration varies from 5 to 500 μm [35]. In our study, the tailor-made electrospun PCL is approximately 200 μm thickness and its porosity is generally 10-20 μm in diameter measured by using Scanning Electron Microscope (SEM) (TM -100 Tabletop Microscope, Tokyo, Japan). Furthermore, fibroblast size is 10-15 μm and SHED or fibroblast-like stem cells are of the same size. Enough porosity is important for proper nutrient and waste transport to facilitate

tissue formation [45,46]. Indeed, Bursac *et al*. [47] found that transportation of nutrient was only limited to diffusion. Scaffold meshes with thickness of greater than 200 μm allows for limited diffusion of nutrients and waste [48]. Pore size normally decreases with decreasing fiber diameter. Average pore size can be as small as 100 nm in some electrospun fiber mats, creating a significant size mismatch between cell size and inter-fiber distance [35]. As a consequence, cellular infiltration, and tissue construction can often be limited due to such small pore size [49]. Some researchers have determined a suitable pore size for cell infiltration and growth based on the cell diameter of which the scaffold is generated [50]. Lowery et al. found that fibroblast cells were capable of bridge pores with diameters of less than 6.5 μm [51]. However, cells extended along single fibers failed to interact with other cells if the pore diameters were greater than 20 μm [52].

Within the limits of this study, surface modification with HA, GE, or HA-GE on electrospun PCL scaffolds demonstrated high biocompatibility and promoted SHED cell adhesion to the mesh surface. However, further research should investigate cell behavior and osteogenic differentiation to determine the potential use of this scaffold for tissue regeneration. All PCL scaffold groups should be selected as they all had strength and weakness in different aspects showed in the experiments.

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