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Manuscript: EJD-2022-5-20 - (2130) - Novel application of 3D scaffolds of poly(e-caprolactone) / graphene as osteoinductive properties in bone defect
Authors: Hendrik Setia Budi (Corresponding Author), Silvia Anitasari (Co-author), Yung Kang Shen (Co-author), Marut Tangwattanachuleeporn (Co-author), Narendra Arya Setiabudi (Co-author)
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Abstract

Objective: Scaffolds provided a surface on which cells could attach, proliferate, and differentiate. Nowadays, bone tissue engineering offer hope for treating bone cancer. Poly(e-caprolactone) (PCL)/graphene have capability as an osteogenic and regenerative therapy. It could be used to produce bone tissue engineering scaffolds. The purpose of this study was to investigate the ability of PCL/graphene to enhance the osteoinductive mechanism.

Materials and Methods: The PCL/graphene scaffold was developed utilizing a particulate-leaching process and cultured with osteoblast-like cells MG63 at 0.5, 1.5, and 2.5 wt% of graphene. We evaluated the porosity, pore size, migratory cells, and cell attachment of the scaffold.

Statistical analysis: Data was expressed as the mean ± S.E.M. and statistical analyses were performed using One-way analysis of variance (*one-way* ANOVA) and Tukey's *post hoc* at a level of *p-value* <0.05.

Results: Porosity of scaffold with various percentage of graphene was non-significance ($p>0.05$). There were differences in the acceleration of cell migration following scratch closure between groups at 24 hours ($p<0.01$) and 48 hours ($p<0.00$). Adding the graphene on the scaffolds enhanced migration of osteoblast cells culture and possibility to attach. 2.5 wt% of graphene exhibited good characteristics over other concentrations.

Conclusions: This finding suggests that PCL/graphene composites may have potential applications in bone tissue engineering.

Keywords: PCL, graphene, scaffold, osteoinductive, tissue engineering

Introduction

Typically, bone reconstruction or regeneration requires the use of a biocompatibility scaffold with a porous structure. The scaffold should possess sufficient strength to support the injured bone in place. A scaffold is generally able to control the proliferation of cells that have migrated from surrounding tissue or been seeded inside the porous surface of the scaffold. Therefore, the scaffold's pore and pore interconnectivity contribute to cell adhesion and proliferation, as well as to the transport of nutrients and oxygen throughout the three-dimensional (3D) constructs [1,2].

Previous studies have reported that scaffolds for bone repair must be biodegradable, biocompatible, porous, and have strong interconnectivity between pores for the cells to adhesion, proliferation, and differentiation [3,4]. Therefore, physical properties like porosity and pore size are important characteristic for the good scaffolds. On the one hand, a high porosity enhances water and nutrient absorption while decreasing mechanical characteristics. Hence, a suitable scaffold's porosity should be comparable to that of bone, such as cancellous bone (79.3). On the other hand, the pore size should regulate the growth of cells that have migrated from the surrounding tissue, which is suitable for cell growth and supporting cell activities such as nutrient uptake and waste processing [5,6,7].

Poly (e – caprolactone) is one of the materials meeting these characteristics (PCL). In recent years, PCL has been utilized as a biomedical scaffold due to its biodegradable and biocompatible properties. However, this material's poor mechanical qualities and pore size, limit its use in bone engineering [2,6]. Therefore, it needs filler that can improve the scaffold's properties [8,9].

Graphene, a new allotrope of carbon, is characterized as a two-dimensional honeycomb lattice composed of carbon atoms in monolayers. Graphene and its derivatives have garnered considerable attention in the field of materials research since graphene has no visible toxicity and exhibits high biocompatibility. Indeed, in vitro experiments have demonstrated graphene's capacity to promote osteoblast proliferation and enhance their differentiation into mature osteoblasts. Thus, graphene is a promising material for enhancing the bio-viability and bioactivity of synthetic scaffolds, especially in cooperation with polymer, such as PCL [9,10,11].

Not only are physical properties an important thing for a good scaffold, but the ability of cells to migrate is important too in a variety of physiological and pathological processes. In vitro cell migration can be studied using the wound healing test (scratch assay), which is a straightforward approach [12]. This method is based on the finding that when an artificial gap is made in a confluent cell monolayer, the cells on the edge of the gap begin to migrate until new cell-cell connections are formed [13,14]. The migratory and differentiation processes of cells are crucial for tissue engineering and regenerative medicine. Recently, the majority of efforts have been directed toward controlling cell destiny via manipulation of biophysical or pharmacological inputs [15]. Chemical cues like ligands, ECM proteins, and biomolecules have all been shown to influence cell activity on chemically changed surfaces [16].

The purpose of this study was to disclose the physicochemical behavior of PCL and graphene at varied concentrations (0.5, 1.5, and 2.5 wt%) on osteoblast-like cells MG-63 in determining which concentrations to enhance the osteoinductive mechanism.

Materials and Methods

Fabrication of PCL/graphene scaffold

Three-dimensional porous scaffolds were manufactured in this study employing a solvent casting/particulate leaching process. PCL (Mn: 80,000) from Sigma-Aldrich (St. Louis, MO, USA) should be used as the matrix material and NaCl as the porogen (Sigma-Aldrich). Graphene was produced by heating a graphite intercalation compound to 700 °C in a common

furnace, positioned in front of a fume closet to avoid inhalation of the nanoparticles, and leaving it there for 60 seconds. These layers grew as a result of ultrasonication, resulting in graphene dispersion in the solvent. For 12 hours at room temperature, polycaprolactone was dissolved in chloroform in a 1:10 w/v ratio. The PCL solution was then added to the NaCl and graphene solutions and stirred for 2 hours with a magnetic stirrer. The blended solution was poured into a mold and allowed to dry at room temperature for one day. Chloroform residues were eliminated during a 24-hour period in a vacuum oven set to 37 °C. The PCL/graphene scaffolds were immersed in deionized (DI) water for 24 hours to eliminate the porogen, with the DI water being replaced every two hours throughout this time period. The PCL/graphene scaffold was then dried in a vacuum oven set to 50 °C for 12 hours. Finally, a porous PCL/graphene-blended scaffold was developed [16]. We synthesized porous scaffolds containing graphene at concentrations of 0.5, 1.5, and 2.5 wt.%. PCL/graphene is manufactured in a 1x1x2 cm^3 format (**Fig. 1**).

Porosity

Dried scaffolds were immersed in absolute ethanol for 2 h and weighed after excess ethanol on the surface was blotted. The porosity was calculated using Equation [5,6]:

$$Porosity = \frac{(M2 - M1)}{\rho V} \quad (1)$$

Where M_1 and M_2 are the mass of scaffolds before and after soaking in absolute ethanol, respectively; ρ is the density of absolute ethanol, and V is the volume of the scaffolds.

Pore Distribution

A scanning electron microscope (Hitachi SU3500) was used to observe samples of 3D porous scaffolds. Gold was sputtered onto samples using a sputter coater in a vacuum chamber and then observed. Then, using Image J software, the SEM picture was analyzed to determine the pore size [5,6].

Scratch Wound Assay (Migration)

The osteoblast-like cells MG-63 were cultured in 12 well plates. Around 3×10^4 cells were seeded into each well and allowed to reach 90% confluency. Using a 200 µL tip, the cell monolayers were scratched and rinsed with PBS to remove detached cells and other debris. Three representative images from each of the scratched areas were photographed to estimate the relative migration of cells. The migration cell (scratch assay) analyzed using inverted miscrope (IX73, Olympus, Japan), 100x magnification and has been processing using Gen 5.0 software. The distance between the two edges of the wound sites was detected at 24 and 48 and analyzed by Image J software. Wound closure was calculated using the formula [17]:

$$Wound\ closure\ (\%) = \frac{(wound\ site\ day\ 0 - wound\ site\ in\ the\ indicated\ day)}{wound\ site\ day\ 0} \times 100\% \quad (2)$$

Cell culture and Morphology

Osteoblast-like cells MG-63 were seeded onto PCL/graphene scaffolds to examine cell adherence and growth characteristics at various graphene weight ratios. First, osteoblast -like cells MG-63 were grown in DMEM (Gibco) with 10% fetal bovine serum (FBS) (Sigma, 12106C) and 1% penicillin (Gibco, 15140122). Cells were cultured in T75 flasks (37 °C with 5% CO2) in a cell culture incubator, and the media was replenished every 2-3 days. Each sample used in the cell culture was 10 x 10 x 2 mm^3 in size. Sterilization of samples was

accomplished by soaking them overnight in 95 percent ethanol and then washing them twice with PBS (Gibco) to eliminate residual ethanol. After that, samples were transferred to 24-well plates. Cells were detached using 0.25 percent trypsin-EDTA (Gibco) and each sample was seeded with 0.5 mL of a cell suspension at a concentration of 10^4 cells/mL in 24-well tissue culture plates. For 21 days, the 24-well plates were incubated in a cell culture incubator. Throughout this period, the medium was renewed every 2-3 days. On days 7 and 21, samples were withdrawn to observe the results of cell culture. A scanning electron microscope was used to determine the cell morphology [16].

Statistical analysis

Data was expressed as the mean ± S.E.M. and statistical analyses were performed using One-way analysis of variance (*one-way* ANOVA) and Tukey's *post hoc* test to determine the relevant data differences using SPSS version 21.0 software (SPSS, USA). Significant differences between groups were determined at a level of *p-value* <0.05.

Results

Porosity and pore size of scaffold

Physical properties, like as porosity and pore size are critical in determining the scaffold's quality. In this study the porosity of PCL incooporated with various concentration of graphene had been similar each other. PCL has an 88% ($\pm$ 1.5). The porosities of the PCL/graphene scaffolds with 0.5, 1. 5 and 2.5 wt%G were 88 ($\pm$ 1.2); 87 ($\pm$ 0.9); 89 ($\pm$ 2.1) respectively (**Fig. 2**). This finding was closed to porosity of cancelous bone ($\pm$ 79.3%).

Pore distribution was determined by a scanning electron microscope (**Fig. 3a**). It showed the porous size of these scaffolds ranged from 0 to 500 μm. The scaffold with concentration graphene 0.5 wt% has a pore size of 0-50 μm larger than others and PCL has a pore size of 400-450 μm larger than others, while the scaffold with a graphene concentration of 2.5 wt% has a pore size of 51-100 μm; 101-150 μm; 151-200 μm; 201-250 μm; 251-300 μm; 301-350 μm; 351-400 μm and 451-500 μm larger than P, 0.5, and 1.5 (**Fig. 3b**).

Migration enhancing of PCL/graphene composite to osteoblast-like cell

In order to evaluate the osteoblast-like cells MG63 migration response, the cells were exposed to different graphene concentrations and allowed to migrate for 24 and 48 hours. Using a wound healing assay, we observed a graphene concentration dependent effect on osteoblast-like cells MG63 migration. The microscopic figure has to be analyzed to obtain information about the migration characteristics of the cultured cells. This could be accomplished manually by measuring the surface area and gap distance using image processing software such as Image J (**Table**). The data shown that the closest 0 µm gap between cells can be achieved on PCL/Graphene 0.5 – 2.5 wt% combination at 48 hours. The farthest cell spacing was found using PCL/Graphene 0.5 wt%.

The condition of 100% cell confluence was utilized to signal that the scratch defect had been completely closed. When treated with 2.5 weight% graphene, osteoblast-like cells MG63 showed nearly full closure of the scratch site within 48 hours, compared to the other treatments. As a result, a statistically significant increase in responsiveness was detected at 2.5 wt% graphene concentrations when compared to the others concentration and control (**Fig. 4**).

Cell adherence on PCL/graphene scaffolds

Cells cultured on PCL and PCL/graphene scaffolds were obtained by SEM on days 7 and 14 (**Fig. 5**). It demonstrated cells adhering to and multiplying within the pore, where each cell has a long filopodia that acts as a signalling pathway between the cells. On day 7, it was discovered that cells had adhered, proliferated, and early differentiated in cell culture. They

were round in shape (1.5 and 2.5 wt% graphene), whereas PCL and 0.5 wt% graphene were flat and elongated. On day 21, differentiation revealed that the majority of cells on the scaffold had spherical forms. In other words, it showed how graphene changes more quickly when there is more graphene than when there is no graphene.

Discussion

The total porosity of the scaffolds was measured in this study using the liquid displacement method. The constructions were more than 85% porous in total. When compared to cancellous scaffolds (which have an average porosity of 79.3%), the porosity of the scaffolds is optimal since it promotes bone ingrowth without reducing mechanical qualities such as strength [7,8].

A variety of scaffolds made from a different of biomaterials and constructed utilizing a variety of fabrication techniques were used. Biocompatibility, biodegradability, mechanical qualities, scaffold architecture, and manufacturing technique are all key factors to consider when creating or establishing the appropriateness of a scaffold for use in tissue engineering. In a successful tissue engineering technique, these properties are deciding criteria when selecting a bio-material (18).

Pore size, along with porosity, is an essential physical property for cell adhesion, gas diffusion, and the delivery of nutrients and wastes. In general, it has been noted that while the scaffold's large pore size or porosity promotes effective nutrition supply, gas transport, and metabolic waste disposal, it results in low cell attachment and intracellular signaling. While a small pore size or porosity may have the opposite effect, the optimal pore size is in the diameter range (100-500 μm), which is thought to stimulate osteogenesis and angiogenesis due to the size of osteoblasts, which is approximately 10-50 μm [9,12,19].

Cell migration and proliferation are required for a range of physiological and pathological processes, including wound healing (20), revascularization, cartilage regeneration, and bone regeneration [21,22]. Cell migration can be induced by biochemical and biophysical cues such as the mechanical properties of matrix, peptides, and growth factors in an immobilized and free form, respectively, which are referred to as mechanotaxis, haptotaxis, and chemotaxis [23]. Two types of biomaterials have been used to facilitate cell migration; one is scaffolds, which have a predetermined architecture, however cell infiltration is more difficult with scaffolds [24]. We assessed an enhancement of osteoblast migration by adding graphene start on 24 h, and closure of the scratch completely within 48 h in comparison to the group of PCL. Graphene better promoted osteoblast migration and seems to have a higher impact of bone tissue regeneration. This is in accordance with a recent study by Du et al, [25].

Because of their osteoinductive properties and antibacterial activity, graphene-based materials (GMs) have a bright future in bone tissue engineering. In bone tissue engineering, GMs trigger osteogenic differentiation via a variety of mechanisms and routes. To begin with, mechanical stimulation from the porous folds of graphene or graphene oxide (GO) can launch a cascade of processes that enhance osteogenic development without the need of chemical inducers. In addition, GMs regulate osteogenesis through the extracellular matrix (ECM), macrophage polarization modulation, the oncostatin M (OSM) signaling route, the MAPK signaling network, the BMP signaling pathway, the Wnt/-catenin signaling pathway, and other pathways (26).

SEM images demonstrate cell attachment, proliferation, and differentiation. It demonstrated that osteoblast-like cells could adhere to, proliferate, and differentiate on the scaffold surface [27]. The proliferation of the cells was regulated by actin. It stimulates signal transduction and cell division. Myosin proteins interact with actin filaments to produce two distinct forms of movement [28,29]. To begin, myosin generates force between actin filaments, causing contractions that pull the rear of moving cells up, pinching cells in half and reshaping them

into tissues [30]. Muscle cells are contracted using a similar manner. Second, myosin associated with subcellular organelles and macromolecular protein and RNA complexes transports these cargos over small distances along actin filaments [31,32].
The rapidly expanding science of tissue engineering for dental tissues will undoubtedly result in a dramatic shift in the availability of novel items for practitioners to employ on a daily basis. When implanted on the side of an injury, a scaffold material should strive for endogenous cell repopulation as well as recipient remodeling.

Conclusions

PCL/graphene composites may have potential applications as novel bone tissue engineering. The porosity and pore size were suitable to induce osteogenesis and angiogenesis, and also stimulated osteoblast migration by adding graphene start from 24 h.

Acknowledgments

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Figures

Fig. 1: Scaffold fabrication. (a) A PCL/graphene scaffold 10x10x2 mm^3 in size; (b) A PCL/graphene scaffold 1x1x2 mm^3 in size.

Fig. 2: Porosity of scaffold with various percentage of graphene; ns (non-significance, $p>0.05$).

Fig. 3: Morphology and pore distribution of PCL and PCL/graphene analysis by SEM. (a) PCL and PCL/graphene scaffold with concentration 0.5, 1.5 and 2.5 wt% graphene. (b) Pore distribution of PCL and PCL/graphene scaffold with concentration 0.5, 1.5, and 2.5 wt% graphene.

Fig. 4: The effectiveness of the PCL/graphene composite was measured by migration cell (scratch assay) using inverted miscrope (IX73, Olympus, Japan), 100x magnification.

Fig. 5: Osteoblast -like cells MG63 attachment to PCL/graphene scaffolds were observed using SEM (Hitachi SU3500). (a) PCL 7 days, (b) PCL 21 days, (c) 0.5 wt% G 7 days, (d) 0.5 wt% G 21 days, (e) 1.5 wt% G 7 days, (f) 1.5 wt% G 21 days, (g) 2.5 wt% G 7 days and (h) 2.5 wt% G 21 days.

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Dear Editor

Dr. Nejdet Adanir, DDS, PhD.

Thank you for giving us the opportunity to submit a revised draft of the manuscript “Novel application of 3D scaffolds of poly(e-caprolactone) / graphene as osteoinductive properties in bone defect” for publication in the European Journal of Dentistry.

We appreciate the time and effort that you and the reviewers dedicated to providing feedback on our manuscript and are grateful for the insightful comments on and valuable improvements to our paper. We have incorporated most of the suggestions made by the reviewers. Those changes are highlighted within the manuscript in red, for a point-by-point response to the reviewers’ comments and concerns.

Thank you very much for your kindness.

Best Regards

Assoc. Prof. Dr. Hendrik Setia Budi, DDS, MDS

Reviewer - 1:	
Comments:	**Response**
Your original research is very interesting and I think it is important topic in bone regeneration which is the scope of this journal. The research's desing and the result are interestingly good. The clarity of writing is good enough and the organization of the paper is well arranged. The SEM figures are remarkable. References are relevant and up to date mostly. Of couse there is a few things should be improve such the clarity of data's interpretation and discussion, the concistency of term, and labelling the figures that you can read in confidential comment. Overall this is a very good article	Thank you for your suggestions and comments. Actually, we have provided the data carefully in the result and explained the data point-by-point in table and figure legends to discuss. According to the comment, we have improved it in the manuscript and be highlighted it in red.

Reviewer - 2:	
Comments-1	**Response**
The topic is very interesting and promising for future novel developments in the field of therapeutic biomaterials. However, they are several flaws in the manuscript that need to be corrected before its publication in the Journal. - The title of the study should mention the study design.	Thank you for your suggestions and comments. We think the title was clear with “what”, “how”, and “how” statement.
Comments-2	
- Please carefully check the use of abbreviations throughout the manuscript, abstract, figures, and tables.	Thank you for your suggestions and comments. We have checked and revised it in the manuscript carefully.
Comments-3	
This abbreviation should be placed right after the Poly(e-caprolactone). Please add more information about the definition of this material.	Thank you for your suggestions and comments. We have revised it in the manuscript carefully.
Comments-4	
Abbreviation should not be placed at the beginning of sentence or simply you can add “The” in front abbreviation.	Thank you for your suggestions and comments. We have revised it in the manuscript carefully.
Comments-5	
What “Mn” abbreviate for?	Thank you for your suggestions and comments. We have revised it in the manuscript carefully. It means is M_n 80,000.
Comments-6	
Cm^3 or mm^3? Should be consistent in PCL/graphene size in other part of this article.	Thank you for your suggestions and comments. We have revised it in the manuscript carefully. It was mm^3
Comments-7	
Reference must be added, otherwise this statement should be put in discussion section.	Thank you for your suggestions and comments. We have revised it in the manuscript carefully and added the references.
Comments-8	
The scaffold with concentration graphene 0.5 wt% has a pore size of 0-50 μm larger than others. You mean “ largest” ?	Thank you for your suggestions and comments. It was not largest

Comments-9	
While the scaffold with a graphene concentration of 2.5 wt% has a pore size of 51-100 μm; 101-150 μm; 151-200 μm; 201-250 μm; 251-300 μm; 301-350 μm; 351-400 μm and 451-500 μm larger than P, 0.5, and 1.5. What "P" abbreviate for?	Thank you for your suggestions and comments. We have revised it in the manuscript carefully. It was the PCL 0.5 and 1.5 wt% graphene.
Comments-10	
Different graphene concentrations. Please describe the graphene concentration.	Thank you for your suggestions and comments. We have revised it in the manuscript carefully. It means 0.5, 1.5, and 2.5 wt%
Comments-11	
In other words, it showed how graphene changes more quickly when there is more graphene than when there is no graphene	Thank you for your suggestions and comments. We have revised it in the manuscript carefully. It was mitosis process. We think, it is clear
Comments-12	
Cancellous scaffolds (which have an average porosity of 79.3%). Or cancellous bone? It is not consistent with this term used in result. Please add the references.	Thank you for your suggestions and comments. We have revised it in the manuscript carefully. It means cancellous bone.
Comments-13	
Two types of biomaterials have been used to facilitate cell migration; one is scaffolds. It is not clear. Please add the exact material of scaffold and describe why chosing this material in this reseach.	Thank you for your suggestions and comments. We have revised it in the manuscript carefully. It means of PCL and graphene.
Comments-14	
Fig. 1: Scaffold fabrication. (a) A PCL/graphene scaffold 10x10x2 mm3 in size; (b) A PCL/graphene scaffold 1x1x2 mm3 in size. The group's name in the figure and tabel must be consistent with the group's name in the article.	Thank you for your suggestions and comments. We have revised it in the manuscript carefully.
Comments-15	
Fig. 3: Morphology and pore distribution of PCL and PCL/graphene analysis by SEM. (a) PCL and PCL/graphene scaffold with concentration 0.5, 1.5 and 2.5 wt% graphene. (b) Pore distribution of PCL and PCL/graphene scaffold with concentration 0.5, 1.5, and 2.5 wt% graphene. Please noted that the SEM's image caption in the PCL section is reversed.	Thank you for your suggestions and comments. We have revised it in the manuscript carefully.
Comments-16	
Fig. 5: Osteoblast -like cells MG63 attachment to PCL/graphene scaffolds were observed using SEM (Hitachi SU3500). (a) PCL 7 days, (b) PCL 21 days, (c) 0.5 wt% G 7 days, (d) 0.5 wt% G 21 days, (e) 1.5 wt% G 7 days, (f) 1.5 wt% G 21 days, (g) 2.5 wt% G 7 days and (h) 2.5 wt% G 21 days. Please add the mark	Thank you for your suggestions and comments. We have revised it in the manuscript carefully.

(asterix, arrow, arrowhead, etc) for the osteoblast-like cells MG63 inside the figure.	

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Date submitted: 2022-06-17

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Thank you,

Sincerely,

Dr. Nejdet Adanir, DDS, PhD.
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Associate Professor, Department of Restorative Dentistry
College of Dentistry, King Faisal University
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