POLY(ε-CAPROLACTONE) MEMBRANES COATED WITH POLY(4-STYRENESULFONIC ACID-CO-MALEIC ACID)-SODIUM SALT ENHANCE OSTEOGENIC PROPERTIES OF PRE-OSTEOBLASTS MC3T3-E1

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SUMMARY

Objectives. Poly (ε-caprolactone) or PCL has been used as the FDA-approved scaffolding material. Despite the biocompatible and biodegradable properties, its low bioactive properties and hydrophobicity restrict the application in bone tissue engineering. This study was conducted in order to improve PCL properties, in terms of supporting osteoblasts function.

Methods. Poly(4-styrenesulfonic acid-co-maleic acid)-sodium salt (PSS-co-MA) was coated on PCL membrane using layer-by-layer technique to fabricate polyelectrolyte multilayer (PEM) films (PCL_PSS-co-MA). The viability and osteogenic functions of pre-osteoblast cell (MC3T3-E1) seeded on material were evaluated, compared to untreated PCL membrane.

Results. MTS assay showed that this PCL_PSS-co-MA films could enhance cell proliferation. RT-PCR results revealed the upregulation of osteogenic markers including Runx-2, OPN and DMP-1 in MC3T3-E1 seeded on PCL_PSS-co-MA films. Alizarin red S staining also showed that MC3T3-E1 cultured on PCL_PSS-co-MA films could generate higher amount of calcium deposition, compared with those on PCL membranes.

Conclusion. The surface modification of PCL membranes by coating with PSS-co-MA PEM films could promote osteoblast proliferation and differentiation *in vitro.* These findings indicated the potential use of PCL_PSS-co-MA membranes as scaffolding material in guided bone regeneration.

Key words: guided bone regeneration, poly (ε-caprolactone), polyelectrolyte multilayer films, poly (4-styrenesulfonic acid-co-maleic acid)-sodium salt, osteoblast functions.

Introduction

Dental implant is widely used as prosthetic replacement in partially edentulous patients. After tooth extraction, bone usually resorbs in a certain pattern. In some cases, osseous defects including crestal dehiscence, apical fenestration, and atrophic ridge occur, resulting in severe bone loss. In all circumstances, adequate amount of bone is needed to support dental implant. Guided bone regeneration (GBR) is a common procedure used to facilitate new bone formation. By using physical barrier membrane to prevent rapid ingrowth of

unwanted epithelial and connective tissue, as well as promote osteogenic cells into osseous defect, this method could improve osteogenesis (1, 2). GBR membranes should be biocompatible, non-toxic and non-immunogenic, while possess optimal mechanical properties. Membranes surface should support osteoblast functions, osteoconduction and osteoinduction. Importantly, it should be bio-degradable, with a degradation rate that is appropriate for supporting bone formation (3).

Poly (ε-caprolactone) or PCL is a synthetic semi-crystalline biodegradable aliphatic polyester with a slow degradation rate similar to the rate of bone regeneration. Its safety has been approved by Food and Drug Administration (FDA, USA) to be used in human as scaffold for tissue engineering of bone and cartilage (4). Although PCL scaffold is biocompatible and biodegradable, it is a hydrophobic and low biological active polymer (4-6). Studies were conducted to modify PCL properties in order that it could better support bone regeneration (5-7).

Polyelectrolyte multilayer (PEM) films can be fabricated through a simple technique which based on an electrostatic layer-by-layer (LbL) self-assembly process by alternated adsorption of polyanions and polycations at material surface. The thickness of PEM films can be controlled and a variety of polyelectrolytes such as DNA, proteins, bioactive molecules and biopolymers can be incorporated. PEM films fabrication is considered an easy, reproducible, inexpensive, fast, stable, and efficient technique to build biologically active surfaces for multiple purposes (8). In recent years, PEM films have been applied to modify the surface properties of biomaterials for promoting new bone formation in bone tissue engineering (9, 10).

Poly (4-styrenesulfonic acid-co-maleic acid) sodium salt (PSS-co-MA) is a copolymer of poly (sodium 4-styrene sulfonate) (PSS) and maleic acid. In our previous study, PSS-co-MA was used in combination with poly (diallyldimethyl-ammonium chloride) (PDADMAC) and PSS to fabricate PEM films [$(PDADMAC/PSS)_4$/PDADMAC+PSS-co-MA] coated on glass surface (11). An increase in osteospecific markers expression of pre-osteoblast (MC3T3-E1) cells cultured on this material including osteopontin (OPN), bone sialoprotein (BSP), osteocalcin (OCN), as well as an elevated alkaline phosphatase (ALP) activity were demonstrated. The *in vitro* calcium deposition rate was also faster than uncoated glass. However, the application of PSS-co-MA PEM films on PCL membranes as barrier membrane for GBR has not been reported.

The objective of this study was to fabricate $(PDADMAC/PSS)_4$/PDADMAC+PSS-co-MA PEM films coating on PCL membranes. The function and behavior of pre-osteoblast cells grown on PSS-co-MA coated surface were evaluated.

Materials and methods

Fabrication of PCL_PSS-co-MA membranes

PCL membranes were fabricated by simple solvent casting method. Briefly, 0.5 g of PCL (M_w = 80,000) was dissolved in 10 ml of 5% w/v chloroform (RCI Labscan, Bangkok, Thailand) at room temperature. The mixture was vigorously stirred for 1 hour and poured onto the 100 mm-diameter glass plate. The mixing solution was solidified by slow evaporation of chloroform under the fume hood at room temperature for 24 hours. PCL membranes were carefully removed from glass plate. PEM films was constructed by layer-by-layer technique to fabricate 9 alternating layers of PDADMAC and PSS, with a final layer of PSS-co-MA[$(PDADMAC/PSS)_4$/PDADMAC +PSS-co-MA] on PCL membranes, as previously described (11). All materials were from Sigma-Aldrich (St. Louis, MO, USA). Chemical structures of the polyelectrolytes used and the fabrication method diagram were shown in Figure 1.

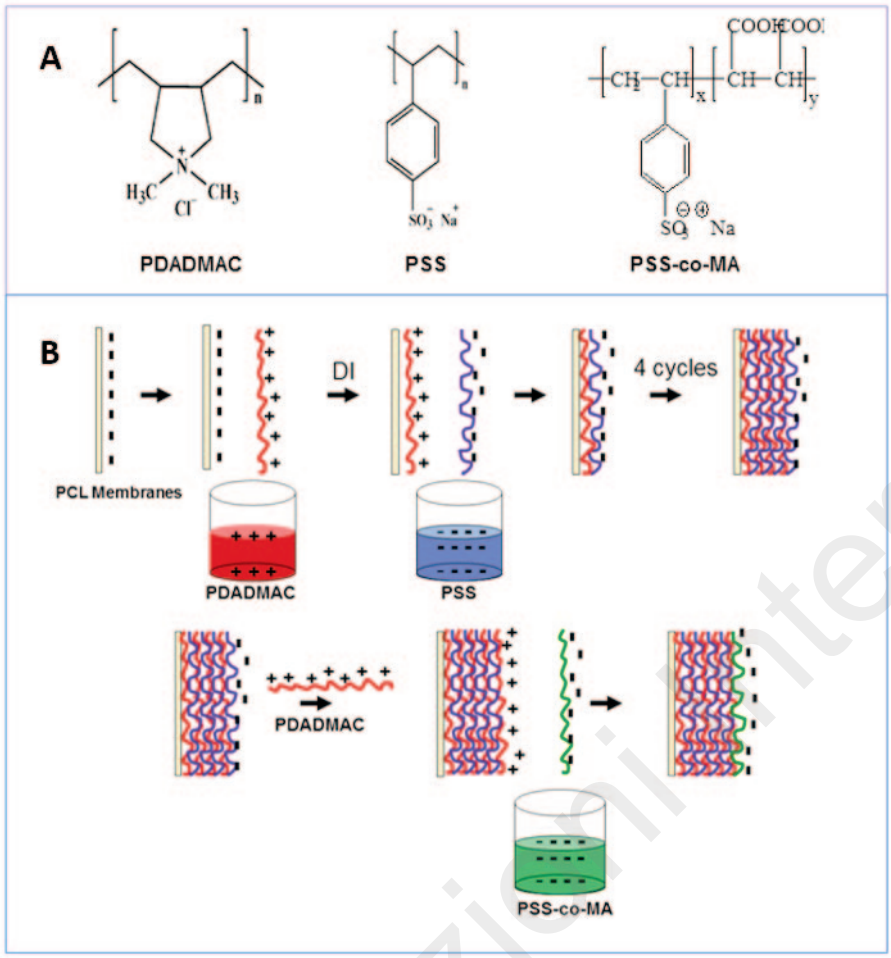

Figure 1
Fabrication of PSS-co-MA PEM film on PCL membranes. A) Chemical structures of the poly-electrolytes used in this study. B) Diagram of (PDAD MAC/PSS) 4/PDAD MAC+PSS-co-MA PEM film fabrication methods on PCL membranes.

Cell culture

The osteoblastic cell line MC3T3-E1 (ATCC CRL-2593) cells were cultured in minimum essential medium (HyQ® MEM/EBSS, Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Gibco™, Grand Island, NY, USA), 1% L-glutamic acid (Gibco) and 1% antibiotic/antimycotic (Gibco). Cultures were incubated at 37°C in humidified atmosphere of 95% air and 5% CO_2. The medium was changed every other day. Cells from passage 12 to 15 were used in the experiments.
PCL and PCL_PSS-co-MA membranes were cut into 10 mm-diameter disc shape. All membranes were sterilized with 70% ethanol for 30 minutes, washed with autoclaved de-ionized water and immersed in culture medium overnight at room temperature. Sterilized membranes or 12 mm round glass cover slips were placed in 24-well tissue culture plate (TCP) and cells were seeded at a density of 40,000 cells per well. Cells cultured on TCP or glass cover slips were used as control.

MTS assay

The viability of MC3T3-E1 cells cultured on TCP, PCL and PCL_PSS-co-MA membranes was determined using MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega®, Wisconsin, USA). After cultured for 0.5, 1, 4, 24 and 48 hours, cells were washed with phosphate buffered saline (PBS) and incubated

with 20% MTS in culture medium (Dulbecco's Modified Eagle's Medium without phenol red; DMEM; Gibco) for 30 minutes. Final products were measured using microplate reader (Varioskan LUX Multimode Microplate Reader, Thermo Fisher Scientific, MA, USA) at 490 nm.

Scanning electron microscopy for cell morphology

MC3T3-E1 cells were cultured on glass cover slips, PCL, and PCL_PSS-co-MA membranes for 0.5, 1, 4, 24 and 48 hours. After incubation period, cells were rinsed twice with PBS and fixed with 3% glutaraldehyde solution (Fluka, Milwaukee, WI, USA) for 30 minutes. The samples were rinsed twice with 0.1 M PBS and dehydrated in a graded series of ethanol (30%, 50%, 70%, 90%, and 100%), followed by critical point dried with 100% hexamethyldisilazane (HMDS; Fluka, Steinheim, Germany) for 5 minutes. After gold sputter coating, the morphology of cell was observed using a scanning electron microscope (Hitachi S-300N, Osaka, Japan).

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis

After cultured for 7 and 14 days, MC3T3-E1 cells were detached from each sample using 0.2% ethylenediaminetetraacetic acid (EDTA, Sigma) in PBS. RNA extraction (Tri reagent, Roche Diagnostics, Indianapolis, IN, USA), reverse transcription (ImProm-II Reserve Transcription System, Promega, Madison, WI, USA), and qRT-PCR (LightCycler® SYBR Green I Master, Roche) were performed according to manufacturer's instruction as previously described (11). The mRNA expressions of OPN, OCN, dentine matrix protein 1 (DMP-1) and runt-related transcription factor 2 (Runx2) were assessed. PCR oligonucleotide sequences of primers used were shown in Table 1. The house keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as control. All experiments were performed in triplicate. The average Cp values and resulting expression ratio of each gene were calculated using LightCycler 480 software version 1.5 (Roche).

Alizarin Red-S staining and calcium quantification

To examine the *in vitro* calcium deposition, MC3T3-E1 cells were seeded at a density of 40,000 cells/well on TCP, PCL and PCL_PSS-co-MA membranes until confluence. Culture medium was changed to medium containing 5 mM glycerol-2-phosphate disodium salt hydrate (β-glycerophosphate; Sigma) and 50 μg ml^{-1} l-ascorbic acid sodium salt (Sigma). Medium was

Table 1 - Primer sequence of oligonucleotides used in qRT-PCR.

Gene	Forward primer (S'- 3')	Reverse primer (5'-3')	GenBank database
Runx2	CGC ATT CCT CAT CCC AGT AT	GGT GGC AGT GTC ATC ATC TG	NM_001145920.1
OPN	CCA ACG GCC GAG GTG ATA	CAG GCT GGC TTT GGA ACT TG	NM_009263.1
OCN	CTT GGG TTC TGA CTG GGT GT	AGG GAG GAT CAA GTC CCG	NM_001032298.2
DMP-1	CAG TGA GGA TGA GGC AGA CA	TCG ATC GCT CCT GGT ACT CT	NM_016779.2
GAPDH	ACT TTG TCA AGC TCA TTT CC	TGC AGC GAA CTT TAT TGA TG	XM_001476723

changed every other day. After cultured for 15 days, alizarin red S staining (Sigma) was performed according to manufacturer's instruction (11). The amount of calcium deposition was quantified colorimetrically by destaining with 10% cetylpyridinium chloride monohydrate (Sigma) in 10 mM sodium phosphate at room temperature for 15 minutes. The optical density was measured using Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific) at 570 nm.

Statistical analysis

Data were analyzed using statistical software (IBM SPSS Statistics 20, IBM, USA). Normal distribution of samples was confirmed by one-sample Kolmogorov-Smirnov test. Data were submitted to one-way analysis of variance (ANOVA) test followed by LSD or Dunnett C *post hoc* tests ($p<0.05$).

Results

Cell attachment and proliferation

The attachment and proliferation of MC3T3-E1 cells cultured on TCP or membranes were evaluated over a range of 0.5 to 48 hours (Figure 2). MTS assay showed similar number of viable cells in all groups at 0.5 and 1 hour incubation. After incubation for 4 hours, the number of viable cells in PCL_PSS-co-MA group was significantly higher than PCL and TCP groups.

Cell morphology

Cell morphology and adherence on each surface were analyzed by SEM imaging (Figure 3). Cells

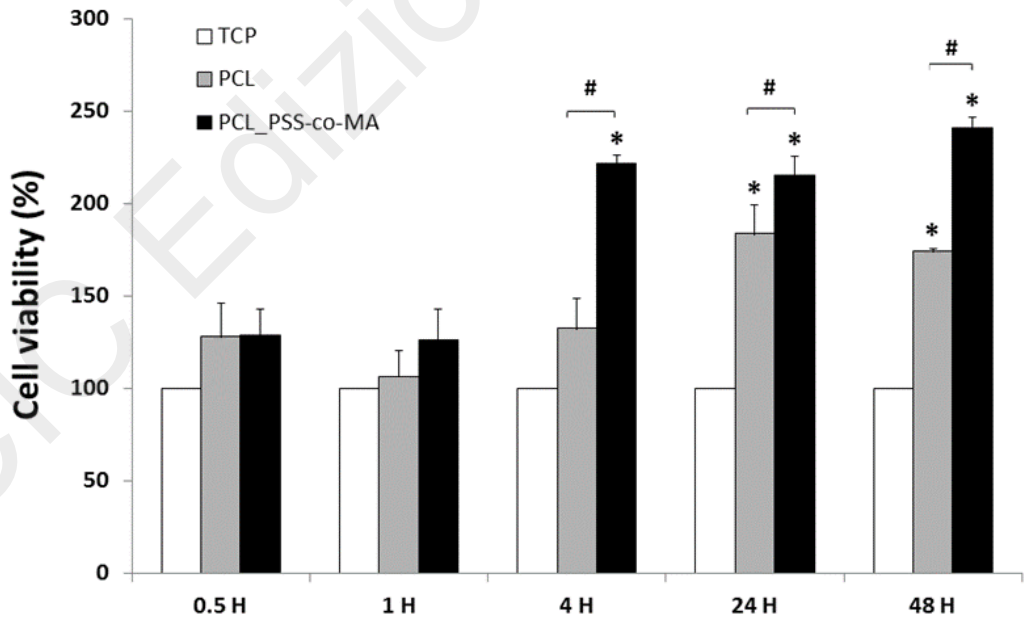

Figure 2
Attachment and proliferation of MC3T3-E1 cells on TCP, PCL and PCL_PSS-co-MA membranes.
The number of viable cells was evaluated by MTS assay after 0.5, 1, 4, 24 and 48 hours of incubation. Cells cultured on TCP were used as control. Results are expressed as mean ± S.D. * Significant difference between TCP group and PCL or PCL_PSS-co-MA membranes group. # Statistical difference between PCL membranes and PCL_PSS-co-MA membranes group, p<0.05.

began to adhere and spread on material surfaces after being seeded. At 30 minutes, cells with circumferential cytoplasmic extension were observed on TCP and PCL membranes, whereas cells with filopodia were displayed on PCL_PSS-co-MA membranes. After 1 hour, cells appeared to be more spreading and flattering, and starting to form contact with the adjacent cells in all groups. After 4 hours, most of the cells were fully spread on all surfaces. Over all, cell morphology, over a range of 0.5 to 48 hours, was not difference in all groups.

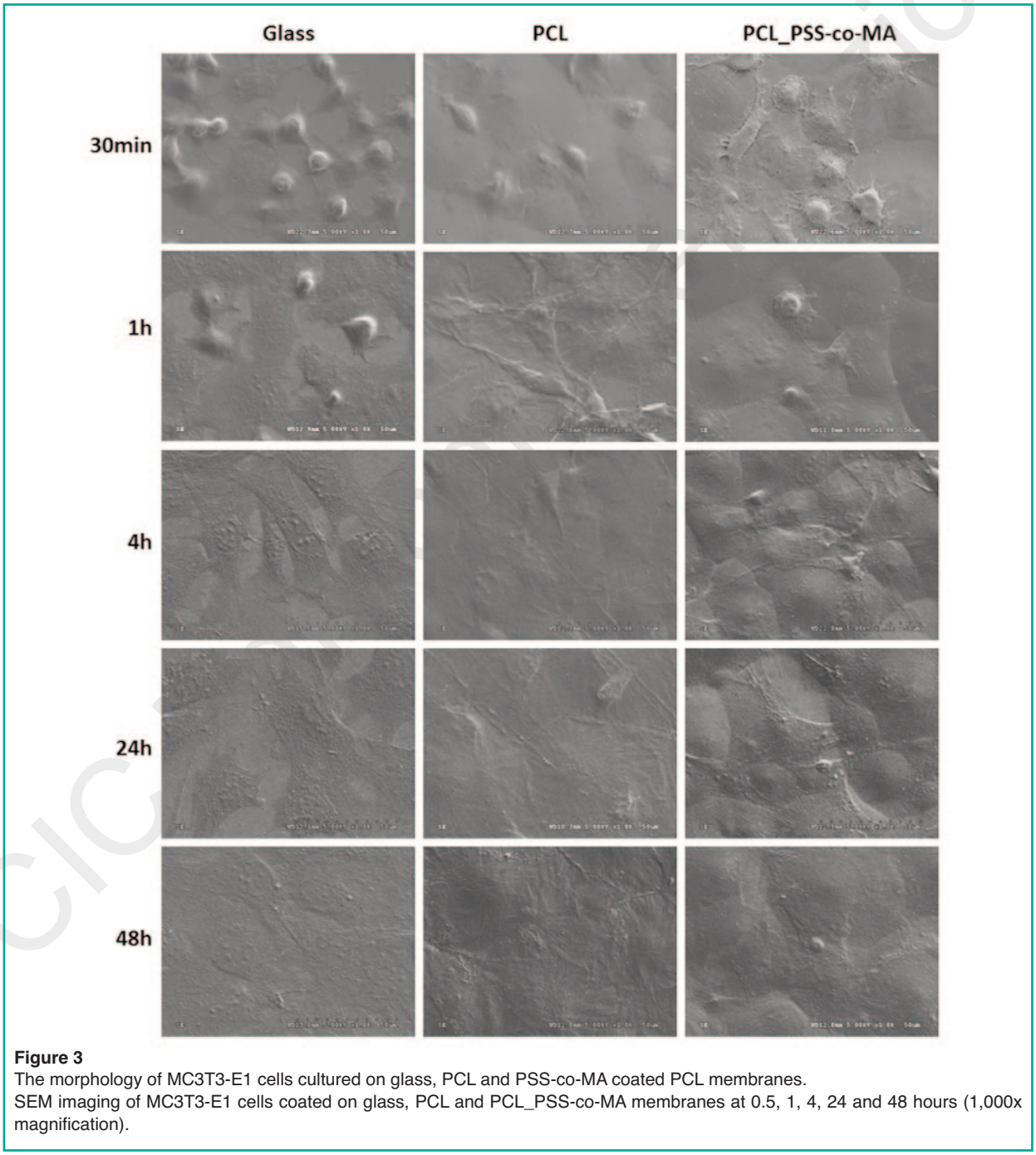

Figure 3
The morphology of MC3T3-E1 cells cultured on glass, PCL and PSS-co-MA coated PCL membranes.
SEM imaging of MC3T3-E1 cells coated on glass, PCL and PCL_PSS-co-MA membranes at 0.5, 1, 4, 24 and 48 hours (1,000x magnification).

Osteogenic gene expression

The expression of osteogenic genes encoding Runx2, OPN, OCN and DMP-1 of MC3T3-E1 cells grown on PCL and PCL_PSS-co-MA membranes were evaluated by qRT-PCR (Figure 4). Although similar Runx2 mRNA levels were detected between cells cultured on PCL and PCL_PSS-co-MA membranes at day 7, the significant increase in Runx2 expression was noted in cell grown on PCL_PSS-co-MA membranes at day 14. Cell cultured on PCL_PSS-co-MA membranes showed highest OCN expression, but with no statistical significance. The expression of OPN and DMP-1 mRNA were significantly highest in PCL_PSS-co-MA group, both on day 7 and 14.

In vitro calcification

The *in vitro* calcification of MC3T3-E1 seeded on each surface was examined after 15 days of culture in osteogenic medium using alizarin red S staining (Figure 5A). The amount of calcium deposition in PCL_PSS-co-MA membranes group was significantly higher than TCP and PCL membranes group (Figure 5B).

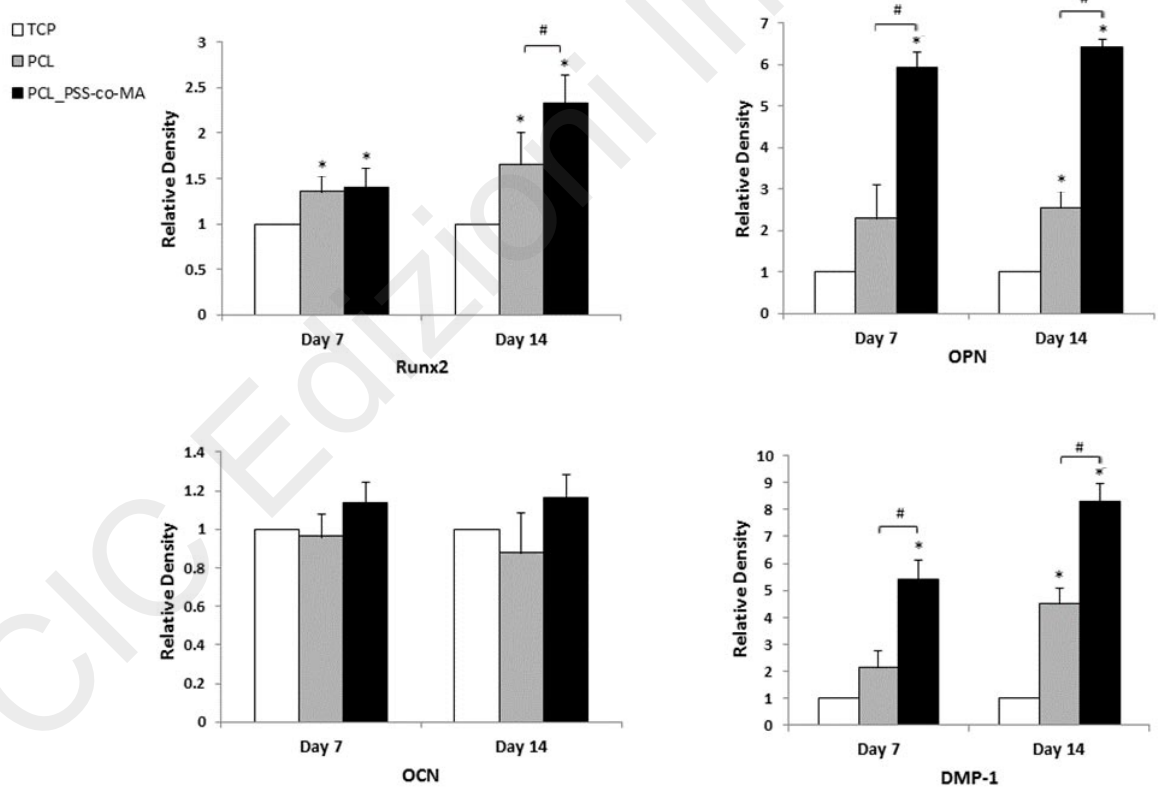

Figure 4
Osteoblastic gene expressions.
The mRNA expression of Runx2, OPN, OCN, and DMP-1, normalized to GAPDH expression, in MC3T3-E1 cells cultured on TCP, PCL and PCL_PSS-co-MA membranes at day 7 and 14. Results are expressed as mean ± S.D. * Significant difference between TCP group and PCL or PCL_PSS-co-MA membranes group. # Statistical difference between PCL membranes and PCL_PSS-co-MA membranes group, $p<0.05$.

Discussion

PCL is one of the most attractive polymers that has been used in bone tissue engineering. The main advantages of PCL over other polymer, such as poly (lactic acid), are that PCL is inexpensive and highly stable in ambient condition (12). Still, the intrinsic hydrophobicity and poor bioactivity of PCL are the main drawbacks that limiting its application in bone tissue engineering. As such, many studies were performed in order to improve PCL properties for supporting bone regeneration (5-7).

In this study, PCL was prepared using simple solvent casting method since it yielded good mechanical strength and achieved favorable properties to support osteoblasts (13). Chloroform was used as solvent instead of acetone, as it leaved PCL membranes smoother surface with less crack. Moreover, more hydrophilic surface and higher mechanical properties could be obtained (14). Since the outermost surface of biomaterial is the first part that contacts with cells or tissue when inserted

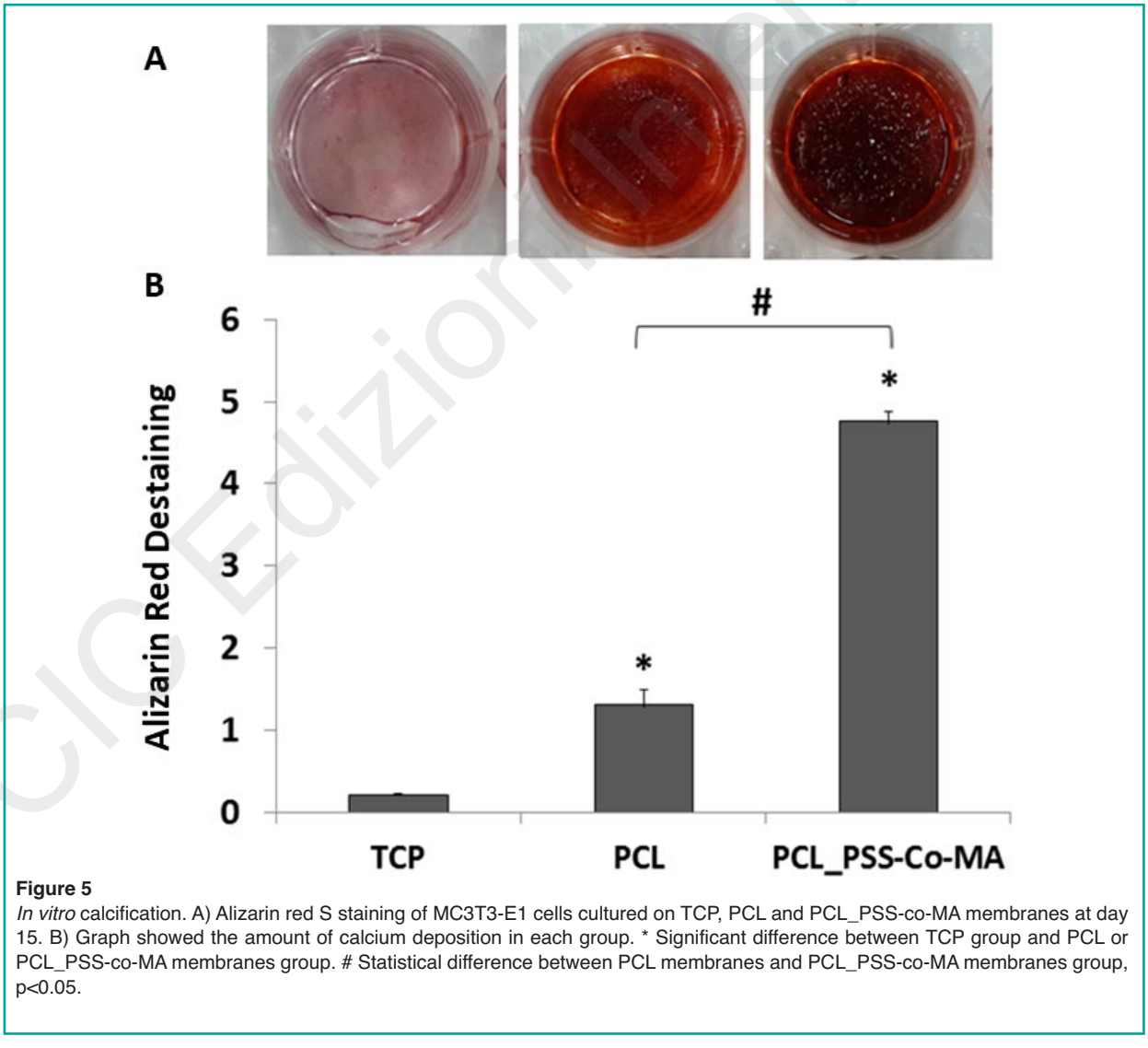

Figure 5
In vitro calcification. A) Alizarin red S staining of MC3T3-E1 cells cultured on TCP, PCL and PCL_PSS-co-MA membranes at day 15. B) Graph showed the amount of calcium deposition in each group. * Significant difference between TCP group and PCL or PCL_PSS-co-MA membranes group. # Statistical difference between PCL membranes and PCL_PSS-co-MA membranes group, $p<0.05$.

into defect site, the surface properties of materials, such as surface roughness, wettability and surface chemistry, are considered key factors in determining initial cellular response as well as the latter events at the cell-material interface, which include cell adhesion, morphological change, functional alteration, proliferation, differentiation and mineralization (15). In this work, PSS-co-MA copolymer was used to modify the surface properties of PCL membranes via PEM films technique, without altering the intrinsic mechanical and chemical properties of the bulk polymer. This method has more advantages in terms of simplicity, production time, and cost. The modified PCL_PSS-co-MA membranes were able to enhance osteoblast cell adhesion, proliferation, differentiation and *in vitro* calcification. The negative charge of carboxyl group on PCL_PSS-co-MA surface might have a positive impact on the hydrophilicity and Ca^{2+} binding capacity of PCL_PSS-co-MA membranes (16-20). Moreover, the maleic acid of PSS-co-MA surface might influence the type and amount of protein adsorptions that is required in the initial step of osteoblast adhesion, proliferation, differentiation and bone formation, such as fibronectin (21-23). Even though PCL_PSS-co-MA membranes could not promote the spreading of MC3T3-E1 cell, the cellular attachment and proliferation rate were improved compared to pure hydrophobic PCL surface. An increase in surface wettability, evaluated by contact angle measurement, of PCL_PSS-co-MA membranes was also observed (data not shown). This was in agreement with our previous work, which demonstrated more hydrophilic properties of glass cover slip coated with PSS-co-MA films over uncoated surface (11). It has been reported that the hydrophobic surface of PCL make it unfavorable for cell growth when it comes in contact with living tissue (24, 25). Several studies suggested that surface wettability is a major parameter affecting cellular behavior at the cell-materials interface, and that cell attachment is better in hydrophilic surfaces than hydrophobic ones (19, 26). In the preparation process of PSS-co-MA solution, the carboxylic acid group in maleic acid could be converted into a carboxylate group and became ionized in this high pH (pH 10) situation, thereby creating a moderate hydrophilic surface (19, 20). Faucheux et al. (19) revealed that strong cellular attachment, spreading, and growth were found in cells cultured on moderate hydrophilic COOH-terminated self-assembling monolayer surface. Moreover, Zhu et al. (25) fabricated moderate hydrophilic PCL surfaces by grafting with poly (methacrylic acid). The improved cellular attachment and proliferation of human fibroblasts over unmodified PCL membrane were also observed.

Another factor influencing osteoblast cell adhesion and proliferation was protein adsorption on membrane surfaces. The changing in surface properties of biomaterials, such as surface wettability and charge or chemical composition, could affect protein adsorption (27). Ma et al. (28) suggested that moderate hydrophilic surfaces were able to adsorb proper amount of protein, which was a key for improving cell-materials interaction. In addition to hydrophilic property, maleic acid on PCL_PSS-co-MA membranes might have an effect on protein adsorption. Maleic acid has been used to improve protein adsorption, such as fibronectin, on material surfaces (22, 23). Fibronectin, an adhesive glycoprotein, has enormous effects on osteogenesis, and plays crucial role in cell adhesion and proliferation. Due to its arginine-glycine-aspartate (RGD) sequence, fibronectin is able to control osteoblast response via integrin-mediated cell adhesion pathway. Moreover, fibronectin is required for osteogenic differentiation and bone nodule formation (21). Fibronectin has been immobilized onto biomaterial surface, and positive effects on osteoblast cell adhesion and function were achieved. For example, Cairns et al. (29) indicated that the adsorbed fibronectin layer on calcium phosphate thin films increased osteoblast cell adhesion, proliferation and promoted osteoblast differentiation. Heller et al. (30)

showed that titanium sheet covalently coated with fibronectin significantly enhanced osteoblast cell adhesion and proliferation, when compared to uncoated titanium sheet.

Osteoblast differentiation consists of three distinct periods; proliferation, extracellular matrix deposition and maturation, and mineralization. During the process, osteoblast cells express various specific extracellular matrix genes, which include Col I, OPN, and OCN, at each time point (31). Additionally, osteoblast differentiation was also regulated by transcription factors such as Runx2, Osterix (Osx) and β-catenin (32). In this study, upregulation of osteogenic markers were found in MC3T3-E1 cultured on PCL_PSS-co-MA membranes, indicating this material could support osteoblast function and bone formation.

Runx2 belongs to the runt-related transcription factors (Runx) family and is a master transcription factor essential in an early stage of osteoblast differentiation (33). Runx2 is involved in regulating the expression of several osteo-specific marker genes such as Col I, OPN, BSP, OCN and DMP-1 in osteoblast (33, 34). OPN, a phosphorylated glycoprotein containing an RGD sequence, mediates cell attachment and cell signaling, as well as regulates the formation and remodeling of mineralized tissues. OPN also interacts with Ca^{2+} and forming a bridge between cells and hydroxyapatite (31, 35). OCN, also known as γ-carboxyglutamic acid or Gla protein, is an abundant Ca^{2+} binding protein and commonly presents in the organic matrix of mineralized tissues such as bone, dentin, and cementum. OCN is tightly bound to hydroxyapatite crystal with very high affinity, and regulates the growth of apatite crystal in the formation of bone and dentin. Both OPN and OCN are highly expressed at or near the time of mineralization (31, 36). DMP-1 is an acidic phosphorylated protein presenting in mineralized tissue and is highly expressed in hard-tissue forming cells including osteoblast, osteoclast and odontoblast. The highly negatively charged molecules and the acidic domains of DMP-1 are involved in the initiation of hydroxyapatite formation in extracellular matrix and/or in the regulation of crystallization process through their very high calcium binding capacity. DMP-1 is found in nucleus during early osteoblast and odontoblast differentiation. Moreover, DMP-1 also acts as a transcription factor in undifferentiated pre-osteoblast to activate osteoblast-specific gene for osteoblast differentiation (34). In the present work, osteoblast cells cultured on PCL_PSS-co-MA membranes showed a significant increase in Runx2 mRNA expression compared to uncoated PCL surfaces. The increased Runx2 expression might lead to the up-regulation of downstream OPN, but did not correlate with the expression of OCN in cells cultured on PCL_PSS-co-MA or PCL membranes. An increased DMP-1 gene expression was also observed in cells cultured on PCL_PSS-co-MA membranes. PSS-co-MA PEM films altered the wettability and chemical composition of PCL surface, which probably affected osteoblast cell adhesion, proliferation, and differentiation. The increased osteogenic gene expression of cell cultured on PCL_PSS-co-MA membranes might partly occur through the effect of maleic acid in PSS-co-MA PEM films on fibronectin adsorption. Our results were in agreement with previous report by Mohamadyar-Toupkanlou et al. (7), which proposed that the presence of nanohydroxyapatite and fibronectin in PCL scaffolds had synergistic effects on promoting cell adhesion and proliferation, as well as osteoblast differentiation in terms of ALP, OCN, OPN, and Runx2 gene upregulation. Moreover, Rapuano et al. (37) revealed that Ti-6Al-4V coated with fibronectin could increase the osteoblast gene expression including Col I, ALP, OPN, BSP, and OCN during the mineralization stage of osteoblastogenesis in MC3T3-E1 cells.

For further assessment, the effect of PSS-co-MA PEM films coated on PCL membranes on an *in vitro* calcium deposition was analyzed using alizarin red S staining. Results showed that the amount of calcium deposition of MC3T3-E1

seeded on PCL_PSS-co-MA membranes was highest in all groups. It has been reported that initial cell adhesion, proliferation and differentiation are responsible for cellular behavior, and are closely correlated with the amount of new bone formation (6). In our study, the highest percentage of cell viability, as well as Runx2, OPN, and DMP-1 mRNA expression in MC3T3-E1 cell seeded on PCL_PSS-co-MA membranes probably accelerated the *in vitro* calcification. Furthermore, the increased mineralization might be from the carboxyl group in maleic acid of PSS-co-MA. As previously mentioned, PSS-co-MA solution was prepared at high pH (pH 10), allowing the conversion of carboxylic acid group into carboxylate group and become ionized, creating the anionic polyelectrolyte surface. The surface functional groups or surface chemistry apparently influenced both osteoblast cell functions and the amount of calcification (17, 38). Tanahashi and Matsuda (38) reported that the negatively charged functional group covering material surface could strongly induce the apatite nucleation, whereas the positively charged group did not, when soaking material in a simulated body fluid (SBF). They suggested that apatite formation and growth were influenced by material surfaces, and the apatite formation was initiate by the interaction of Ca^{2+} and the surface functional groups. This event might occur in the calcification process of implanted materials, where ionic species are directly in contact with human body fluids (38). The carboxyl terminal group formed on polymer surface might have a role in inducing the nucleation and promoting the apatite formation on polymer surface in SBF (16, 17). The mechanism of apatite nucleation and growth on polymer surface terminating with carboxyl groups was proposed (17, 39). First, the negatively charged carboxyl groups were initially bound with the positively charged calcium ions ($-COO^- Ca^{2+}$) via an electrostatic interaction, which triggered the initial formation of apatite nucleation. The negatively charged phosphoric ions were further attracted to ($-COO^-Ca^{2+}$), forming calcium phosphate nucleation. After the apatite nuclei formation, spontaneous growth occurred through the uptaking of calcium and phosphate ions from surrounding fluid until the whole polymer surface was completely covered with primary layer of apatite crystals. Moreover, higher amount of carboxyl group on polymer surface was found to increase the available calcium phosphate nucleation site, as well as apatite mineral growth rate in SBF (16-18). The nucleation and growth of a bone-like apatite on material surface was influenced by several factors such as the charged density of surface functional groups, interaction intensities among the corresponding ionic groups and/or polar groups, and accessibility of these corresponding groups (17, 40). In all, it can be stated that the negatively charged carboxyl group of maleic acid might be important for improving osteoblast functions and calcium phosphate formation on PCL_PSS-co-MA membranes.

In this work, PSS-co-MA PEM films were fabricated on PCL membranes to improve the osteogenic properties. Results showed that PCL_PSS-co-MA membranes could enhance cell proliferation, osteogenic gene expression and *in vitro* calcification of pre-osteoblast MC3T3-E1 cells. Within the limitation of this study, it was suggested that PCL_PSS-co-MA membranes might be used as a barrier membrane that could potentiate osteoblast functions and improve new bone regeneration. However, the application of PCL_PSS-co-MA membrane still faces many challenges, such as the control of degradation rate, which required further investigations.

Acknowledgements

This work was financially supported by "Young Researcher Development Project", Khon Kaen University. The Authors would like to thank Assoc. Prof. Dr. Ponlatham Chaiyarit for his advice in this project.

Conflicts of interest statement

The Authors declare no conflicts of interest relevant to this study.

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