



Bioactive surface modification of polycaprolactone using MG63-conditioned medium can induce osteogenic differentiation of mesenchymal stem cells

Chih-Hsin Lin¹, Ming-Chau Chang^{2,4}, Shih-Chieh Hung^{3,4}, Shyh-Yuan Lee^{1,5}, and Yuan-Min Lin^{1,5,*}

¹Department of Dentistry, School of Dentistry, National Yang-Ming University, No. 155, Sec. 2, Linong Street, Taipei 112, Taiwan, ROC

²Faculty of Medicine, Institute of Anatomy and Cell Biology, National Yang-Ming University, Taipei 112, Taiwan

³Stem Cell Laboratory, Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei 112, Taiwan

⁴Orthopaedics and Traumatology, Taipei Veterans Hospital, Taipei 112, Taiwan

⁵Department of Stomatology, Taipei Veterans Hospital, Taipei 112, Taiwan

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ABSTRACT

We demonstrated an approach that modifies the scaffold surface with a range of molecules, simultaneously conjugated to the scaffold by a single treatment with concentrated conditioned medium (CM), inducing mesenchymal stem cells (MSC) to differentiate into osteogenic lineages. We first show that the CM from MG63 cells is capable of inducing the desired MSC differentiation over 7 days. We then analyze how the biodegradable polymer polycaprolactone (PCL) can be used as the scaffold. Using a CO₂ plasma treatment, it is possible to conjugate MG63 CM proteins onto the PCL film surface, and we show a gradual release of protein from such a modified PCL scaffold. Finally, we verified cell differentiation and marker expression of MSCs grown on the modified PCL and show that osteogenic markers, including alkaline phosphatase and Runx2 mRNA, are significantly upregulated. Immunostaining also shows a strong expression of the Runx2 protein. Our study shows that the differentiation effects of a condition medium can be preserved when its content is used to modify the surface of polymer scaffolds. This approach may be further applied for the differentiation of various cell lines, and it provided a first step toward growing MSCs on more complex scaffold shapes aimed at therapeutic uses.

Address correspondence to E-mail: ymlin@ym.edu.tw

Introduction

A common tissue engineering approach is the conjugate bioactive molecules onto the scaffold surfaces in order to enhance cell attachments, proliferations, and differentiation on the scaffolds. Some common scaffold materials, such as PGA, PLA and PCL, have been modified with various proteins or peptides [1–3].

PCL is a common biodegradable polyester. It can degrade into smaller molecules through the breakage of the ester bonds over a several months period. Except for the terminal hydroxyl groups, there are no functional groups on the PCL backbone, making it very difficult for further conjugation. A simple approach to introduce functional groups on the scaffold surface is the low-temperature plasma treatments. The reactive species in the plasma can react with the scaffold surfaces and generate function groups. For example, carbon dioxide plasma can introduce $-\text{COOH}$ groups onto the surface of PCL films. To conjugate biomolecules with $-\text{COOH}$

groups of the PCL surfaces, the zero-length cross-linker EDC can be used to react with the carboxylic acid first, forming a semi-stable intermediate ester, and then the intermediate ester can further react with the primary amines of the biomolecules.

Bone marrow-derived mesenchymal stem cells (MSCs) are a good model for the study of the surface bioactive modification on the cell differentiation. Because MSCs have the ability to differentiate into several different lineages, such as osteoblasts, chondrocytes and adipocytes [4], they have wide applications across the field of tissue engineering [5]. It is relatively easy to manipulate MSCs to differentiate into osteoblasts, and many studies have demonstrated MSC-induced osteogenesis across a range of biomaterials used in tissue engineering applications [6–8]. A typical osteogenic induction medium (OIM) contains minimum essential medium (MEM), fetal bovine serum (FBS), dexamethasone, ascorbic acid, and glycerophosphate. The purpose of these ingredients is to stimulate alkaline phosphatase activity, encourage

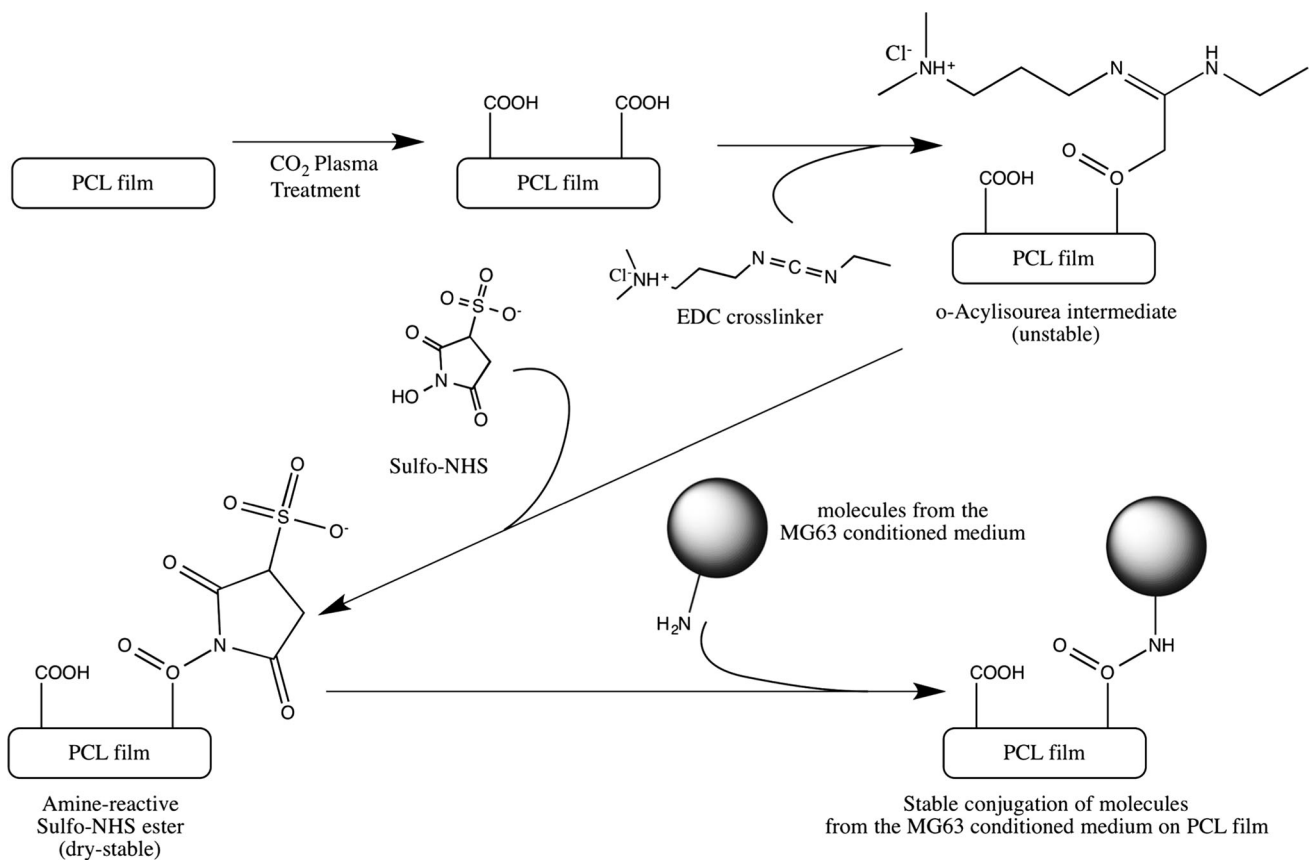


Figure 1 The schematic of the surface modification reaction on a PCL films using molecules from MG63-conditioned medium.

calcium deposition, and to express important osteogenic markers [9, 10].

To differentiate MSCs used for regenerative medicine research, traditional protocols typically require 14 days and frequent changes of the OIM. Our goal is to develop methods that make this process easier, in order to allow MSC differentiation to be performed in a more streamlined and manufacturable way. Many studies have previously reported that conditioned media (CM) contain a range of components that regulate cell functions and that its components contain important growth factors and cytokines that induce cell proliferation and regulate signal pathways [11–13]. Moreover, CM also stimulates differentiation of stem cells into cell lineages, such as endothelial cells or astrocytes [14, 15].

For the choice of cell types for CM, we considered MG63 cells. MG63 is a type of osteosarcoma cell [16]. It was reported that conditioned media from MG63 contains bone morphogenetic proteins (BMPs) [17]. These BMPs are growth factors that are members of the transforming growth factor beta (TGF- β) superfamily, and they can regulate molecular signaling pathways that result in important osteogenic stimulation factors [18]. They are therefore a potent inducer of MSC differentiation [19–21]. Runt-related transcription factor 2 (Runx2) also plays an important role in osteogenic differentiation and skeleton development [22]. Runx2 transcripts are able to stimulate several osteogenic signaling pathways, such as osteocalcin (OCN), collagenase III, type I collagen (Coll), and alkaline phosphatase (ALP) activity [23–26]. Moreover, BMPs are well known to promote Runx2 expression by activating the p38, ERK1/2, JNK1/2, and other signaling transduction pathways that can stimulate the osteoblast phenotype [27, 28]. Therefore, it may be possible to induce the differentiation of MSC cells into osteogenic lineage using the MG63-conditioned medium.

In this study, we will first identify a suitable CM that can induce MSC differentiation. We then establish a method to create bioactive surface modifications on the PCL film surfaces (Fig. 1), so that differentiation of MSCs can take place directly on the scaffold and without the need for further manual interaction with the sample. We then verify that the differentiated cells show osteogenesis and express relevant osteogenic markers.

Materials and methods

Sample preparation and PCL treatment

Culturing of cells

Human bone marrow mesenchymal stem cells (MSCs) were kindly provided by Dr. Chang's lab from Taipei Veteran General Hospital (Taipei, Taiwan). MSCs were cultured in α -MEM supplemented with 15% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin, and seeded in a 10 cm plastic dish. Cells were subcultured after trypsinization and used in experiments until passage 4. Human MG63 osteoblast-like cells were cultured in DMEM medium with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. The culture medium was replaced every 3 days. After trypsinization, cells were subcultured or used for the following experiments.

Collection, lyophilization, and dialysis of MG63-conditioned medium

Once the MG63 cells reached 70% confluence, we replaced the culture medium with basal DMEM without serum and incubated the cells at 37 °C for 24 h. The resulting CM was collected, and its volume measured as our 100% concentration reference. We then centrifuged at 1500 rpm for 5 min, and filtered supernatant with a 0.22 μ m filter. After freeze drying the samples overnight, they were loaded into dialysis tubing (MWCO 6000–8000) and stored at room temperature for 48 h, with the dialysis buffer exchanged every 1–2 h. After 48 h, we removed the media from the tubing. We then freeze dried the samples again, creating a dry powder. From this powder, and using the reference volume from the original CM collected, we mix the appropriate amounts of water and powder to create CM at 25, 50, 100%, 10 \times , 50 \times , and 100 \times of the original concentration.

PCL synthesis and surface plasma treatment

PCL with weight average molecular weight of around 50,000 Dalton was synthesized by ring-opening polymerization of ϵ -caprolactone at 130 °C with catalyst stannous octoate. The synthesized PCL was solvent-casted into circular films in wells of 6-well plates or 24-well plates. The films were then

Table 1 Sample abbreviation

Sample name	Conditions for differentiations or sample preparations
Control	MSC culture medium in petri dish
OIM	OIM in petri dish
25% CM	25% diluted CM in petri dish
50% CM	50% diluted CM in petri dish
100% CM	CM as collected in petri dish
PCL	Unmodified PCL film prepared by solvent-casting
PCL-2 m	PCL \Rightarrow CO ₂ plasma (2 min)
PCL-2 m-EDC	PCL \Rightarrow CO ₂ plasma (2 min) \Rightarrow EDC
PCL-2 m-1 \times CM	PCL \Rightarrow CO ₂ plasma (2 min) \Rightarrow EDC \Rightarrow CM (1 \times)
PCL-2 m-1 \times CM-UV	PCL \Rightarrow CO ₂ plasma (2 min) \Rightarrow EDC \Rightarrow CM (1 \times) \Rightarrow UV ster for 30 min
PCL-2 m-1 \times CM-2 m	PCL \Rightarrow CO ₂ plasma (2 min) \Rightarrow EDC \Rightarrow CM (1 \times) \Rightarrow CO ₂ plasma sterilization for 2 min
PCL-2 h-10 \times CM-2 m	PCL \Rightarrow CO ₂ plasma (2 h) \Rightarrow EDC \Rightarrow add CM (reconstituted at tenfolds) for conjugation \Rightarrow CO ₂ plasma sterilization for 2 min
PCL-2 h-50 \times CM-2 m	PCL \Rightarrow CO ₂ plasma (2 h) \Rightarrow EDC \Rightarrow add CM (reconstituted at 50 folds) for conjugation \Rightarrow CO ₂ plasma sterilization for 2 min
PCL-2 h-100 \times CM-2 m	PCL \Rightarrow CO ₂ plasma (2 h) \Rightarrow EDC \Rightarrow add CM (reconstituted at 100 folds) for conjugation \Rightarrow CO ₂ plasma sterilization for 2 min

EDC in this table stands for PCL pretreatment of its carboxylic acid with EDC and NHS. For cells cultured on PCL films or modified PCL films, standard MSC culture medium was used for differentiation

treated with CO₂ plasma using a 50 w 40 kHz low-frequency plasma reactor (Diener Femto, Germany) for 2 min to 2 h. The flow rate of the plasma is 10 ml/min. Some PCL films were left untreated for XPS and surface contact angle measurements.

Conjugation of lyophilized CM proteins on the PCL films

All conjugation reactions were performed in two steps. In the first step, we treated the surface carboxylic acid of the PCL films with an activation buffer containing 5 mM NHS, 2 mM EDC, 0.1 M MES, and 0.5 M NaCl at pH 5.5 for 15 min. In this step, semistable amine-reactive NHS esters were formed on the PCL surface. In the second step, the activation buffer was aspirated first and a coupling buffer, which contained concentrated MG63-conditioned medium in PBS, was added onto the activated PCL films. In this step, the primary amines of the molecules in the CM were conjugated to the PCL surfaces. These reactions were performed at a pH 7 for 2 h with continuous shaking on an orbital rocker. The schematic of the PCL surface modification is illustrated in Fig. 1.

Sterilization procedures

Prior to seeding the MSCs onto the modified PCL films, the PCL films were sterilized. Because different

sterilization methods can affect the conjugated proteins on the PCL surface, we evaluated two different sterilization methods, including UV and CO₂ sterilization. For UV sterilization, modified films were exposed to 0.12 J/cm² of 254 nm UV light for 30 min (Vilber Lourmat BLX254). For plasma sterilization, modified PCL films were treated with the CO₂ plasma again for 2 min.

MSC differentiation using OIM, CM, and CM-conjugated PCL films

The MSC differentiation conditions and their abbreviations are shown in Table 1. For all the samples, the differentiated period was 7 days. MSCs were differentiated either in different differentiation media in petri dish or in MSC culture medium on PCL or conditioned-medium-modified PCL surfaces. The experimental control was the cells differentiated in MSC culture medium in petri dish (abbreviated as control, as shown in Table 1). For positive control, osteogenic induction medium (OIM) was used as the differentiation medium. OIM consists of α -MEM supplemented with 15% fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin, 10 nM dexamethasone, 50 μ g/ml ascorbic acid-2 phosphate, and 10 mM -glycerophosphate stock solution. During

the 7-day differentiation period, OIM was changed three times a week.

Sample analysis and data collection

Surface elemental analysis using XPS (X-ray photoelectron spectrometer)

PCL itself does not contain nitrogen or sulfur, but the CM proteins conjugated onto PCL contain these elements. Therefore, we use an XPS to analyze the chemical surface composition for each of the modified PCL film types in order to assess whether conjugation has taken place. For all of the samples, both survey and high-resolution spectra were recorded. The latter allows for a precise evaluation of the chemical composition. We used an asymmetric background subtraction algorithm (Shirley's algorithm) prior to curve fitting the peaks and determining the atomic percentages. Because plasma sterilization may damage the conjugated proteins on the material surface, surface elements of the modified PCL films after CO₂ sterilization were also characterized by XPS.

Water contact angle θ_w measurement

The water contact angles were determined using a sessile drop shape analysis system (Tantec, Germany) at 21 °C. A minimum of eight measurements were taken for each film type we characterized.

Protein release test

To verify the conjugation of proteins onto the PCL, and to evaluate their subsequent release, released protein over 1 week period was measured using a BCA Protein Assay (Merck Millipore, CA), following the manufacturer's protocol and using bovine serum albumin as a standard. Protein presence was measured by absorbance at a wavelength of 562 nm, using a Tecan Sunrise ELISA plate reader (Männedorf, Switzerland).

MTT cell proliferation assay

An MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl-tetrazolium Bromide) cell proliferation assay was performed, to measure the mitochondrial activity spectrophotometrically of the differentiating MSCs.

The MSCs were differentiated in the different medium, unmodified PCL, and CM protein-modified PCL. The MTT assay was performed according to manufacturer's protocol (Sigma, St. Louis, MO, USA).

Evaluation of osteogenic marker expression, measuring mRNA with real-time PCR

The total cellular RNA was extracted from MSCs using TRIzol reagent (Invitrogen, Carlsbad CA, USA). Complementary DNA (cDNA) was then reverse-transcribed with 1 µg total RNA and an oligo-dT primer, using a Superscript II kit (Invitrogen, Carlsbad CA, USA). Real-time PCR was carried out on an iCycler iQ real-time detection system (Bio-Rad, Hercules, CA) with SYBR-Green I (stock solution 25000×) as the fluorescent dye. Specificity was confirmed by melting curve detection following the real-time PCR. Cycling conditions were 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. For quantification, the target gene was normalized to the GAPDH internal standard gene. The primers for the real-time PCR were designed using the Beacon Designer 2 software (PREMIER Biosoft International, Palo Alto, CA). All gene-specific oligonucleotides were primers of human GAPDH, Osteopontin, Runx2, or ALP. Specifically, the sequences were, respectively: GAPDH (5'-AAGGT GAAGGTCGGAGTC-3' and 5'-TGTAGTTGAGGT CAATGAAAGG-3'), Osteopontin (5'-GGAAA GCGAG GAGTT GAATG-3' and 5'-GTGGG TTCA GCACT CTGGT-3'), Runx2 (5'-GCAGT TCCCA AGCAT TTCAT-3' and 5'-CACTC TGGCT TTGGG AAGAG-3'), and ALP (5'-GACAA GAAGC CCTTC ACTGC-3' and 5'-GACGT AGTTC TGCTC GTGGA-3').

Immunostaining of differentiated MSCs

After 7 days of differentiation, MSCs were fixed and stained with Osteopontin (SC-73631; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Runx2 antibodies (SC-12488; Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by incubation with a secondary antibody (DAKO, Carpinteria, CA, USA), and then cells were mounted on glass slides and the fluorescent images were recorded.

Table 2 XPS surface element analysis

Element	PCL	PCL-2 m	PCL-2 m-1×CM	PCL-2 m1×-CM-2m
Carbon	74.29%	72.21%	52.88%	63.12%
Nitrogen	n/d	n/d	1.39%	1.30%
Oxygen	25.71%	27.79%	44.66%	34.82%
Sulfur	n/d	n/d	1.07%	0.76%

Summary of the atomic concentrations for different samples. Elements not detected in the XPS are noted as n/d. Unmodified PCL only contains carbon and oxygen, while modification with CM indicates additional presence of nitrogen and sulfur, as a consequence of CM protein conjugation. Measured values reported as mean, with $n = 3$

Statistical analysis

All experiments were performed at least three times, and the data we present are expressed as the mean and standard deviation. An analysis of variance (ANOVA) was performed using SPSS Statistics Desktop 20 (IBM, Armonk, NY, USA), and a value of $p < 0.05$ was considered statistically significant. In the figures to follow, we use the symbol ** to denote samples for which $p < 0.001$ when compared against PCL or glass (Fig. 1), and we use the symbol * to denote those samples for which $p < 0.01$. When comparing the results against OIM (Fig. 4), for emphasis and clarity, we use the symbols ## and # instead.

Results

Surface composition of the modified PCL

Using X-Ray photoelectron spectroscopy (XPS), we found no presence of nitrogen or sulfur in unmodified PCL and PCL-2 m, as shown in Table 2. The presence of nitrogen and sulfur can be observed in the PCL-2 m-1×CM and PCL-2 m1×CM-2 m. The latter contains slight lower nitrogen and sulfur percentage, suggesting the CO₂ plasma can clean and remove the conjugated proteins.

Hydrophilicity of modified PCL: contact angle measurement

All surface-modified samples showed lower θ_w than unmodified PCL (Fig. 2). After CO₂ plasma treatment, the θ_w of PCL-CO₂ decreased dramatically from 60° to 23°. When the PCL-CO₂ interacts with EDC to form a semi-stable intermediate, its θ_w increases from 23° to 44°. However, θ_w decreases

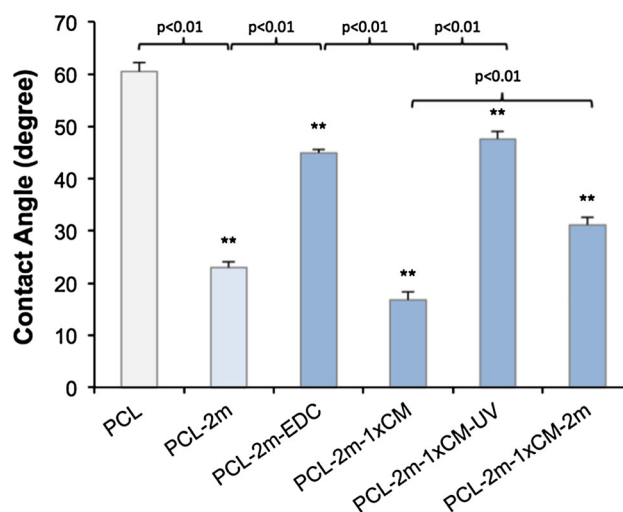


Figure 2 Measured water contact angles for unmodified PCL films and PCL films with different modifications. All modified PCL films has significantly lower contact angles than the unmodified PCL with $p < 0.01$ (labeled as **). After UV and CO₂ plasma sterilization, the contact angle significantly increased, suggesting that these two sterilization steps change the conjugated proteins.

remarkably to 16° for the conditioned-medium-conjugated PCL (PCL-2 m-1×CM). After treating the PCL-2 m-1×CM sample with UV light, θ_w increased to 47°. If we used CO₂ plasma sterilization instead (PCL-2 m-1×CM-2 m), the sample showed a θ_w of 31°, which is less than θ_w of UV-sterilized samples.

In vitro protein release study

In order to study protein release of the modified PCL scaffolds, we treated samples with CO₂ plasma for 2, 30 min, and 2 h. We then used a BCA Assay to measure the concentration of released proteins. Figure 3a shows the cumulative protein release as a function of time, and Fig. 3b shows the daily released quantity measured. Samples treated with CO₂ plasma

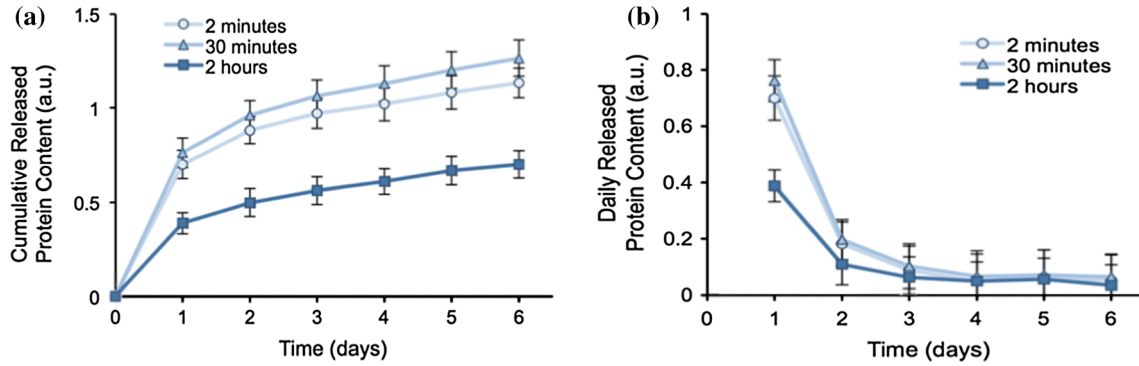


Figure 3 Protein released as a function of time for different CO₂ plasma process durations, showing the cumulative release (a) and the daily measured release (b). After an initial boost on day 1, release drops but is stable for the remaining days. Plasma exposure

for 2 and 30 min resulted in essentially similar characteristics, while 2 h plasma exposure resulted in a much reduced day 1 boost with a continued release thereafter.

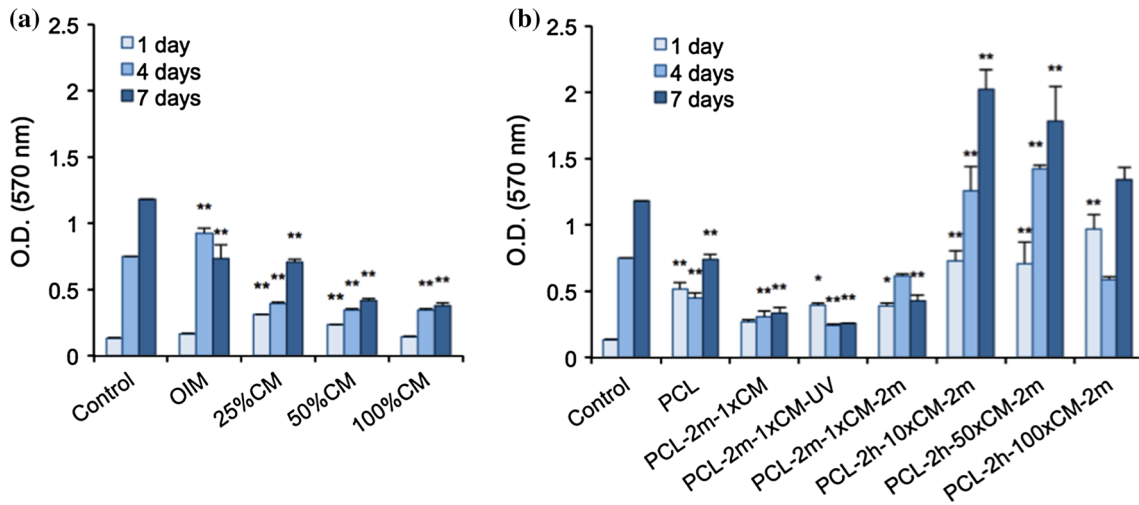


Figure 4 Comparison of the cell proliferation assay MTT for 1, 4, and 7 days in different media (panel a), and on different CM-modified PCL films (panel b), against a control tissue culture petri dish (TCP). In OIM and all concentrations of CM, the cell proliferations were lower than the control (panel a), the MSC

culture medium. Higher cell proliferation than control was observed for PCL modified with concentrated CM (10×, 50× and 100× of CM, panel b). Asterisk and double asterisk stand for significant difference between samples and the control with $p < 0.05$ and 0.01 , respectively.

for 2 and 30 min show very similar release profiles. During the first day, we see a burst of protein released, followed by a much slower and constant release of proteins over the next 6 days. For the samples that received 2 h plasma treatment, the release curve has a similar shape but shows an overall lower protein release (Fig. 3a). However, we note that the treatment duration primarily impacts the initial release burst, while it has much less impact on the amount of protein released gradually on day 2 and onwards (as shown in Fig. 3a by the parallel curves, or Fig. 3b). These results show that for all treatment protocols, we see a gradual release of

proteins from the modified PCL scaffolds, with notable differences in the initial protein released.

Cell viability: MTT cell proliferation assay

The MTT assay was used to evaluate the metabolic activities of MSCs differentiated in different media in petri dish (Fig. 4a) and in standard MSC culture medium on different surfaces (Fig. 4b). A lower MTT value than the control (TCP) was observed for cells treated with OIM, 25% CM, 50% CM, and 100% CM (Fig. 4a). In Fig. 4b, we can observe the higher

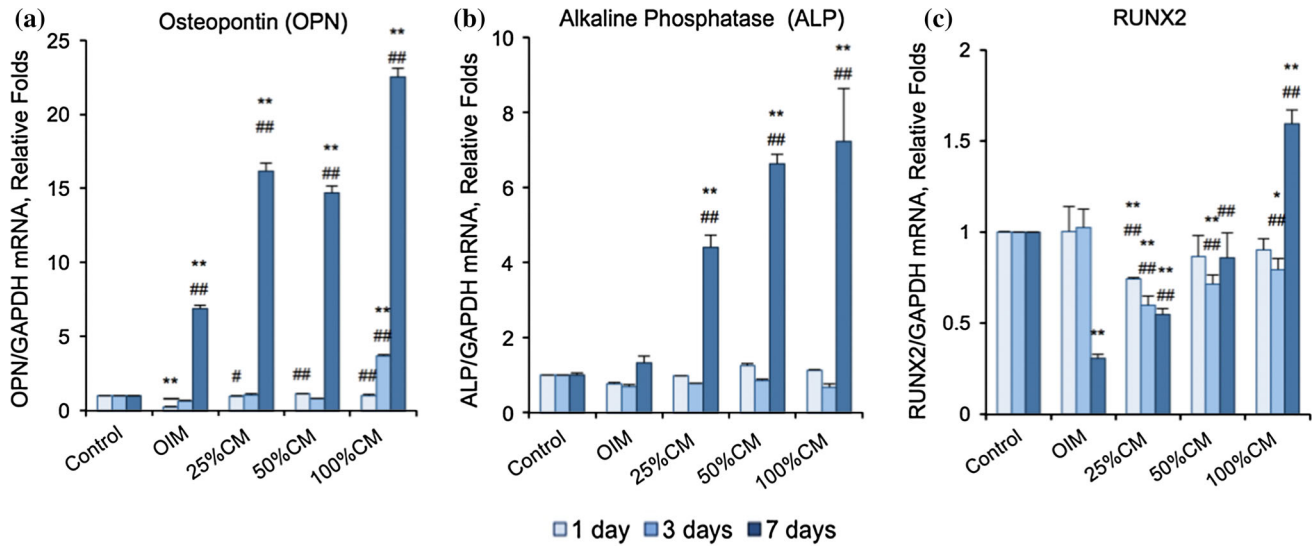


Figure 5 Effects of CM media treatment on the expression of the osteogenic differentiation makers OPN, RUNX2, and ALP, respectively, after 1, 3, and 7 days. We observed a significant increase of OPN toward day 7 (panel a) with the increasing CM concentration. Increasing CM concentration again showed a notable increase in ALP expression toward day 7 (panel b). For

RUNX2 (panel c), we did not see a significant increase in marker expression with the exception of 100% CM on day 7. Asterisk and double asterisk stand for significant difference between samples and the control with $p < 0.05$ and 0.01 , respectively. # and ## stand for significant difference between samples and the positive control OIM with $p < 0.05$ and 0.01 , respectively.

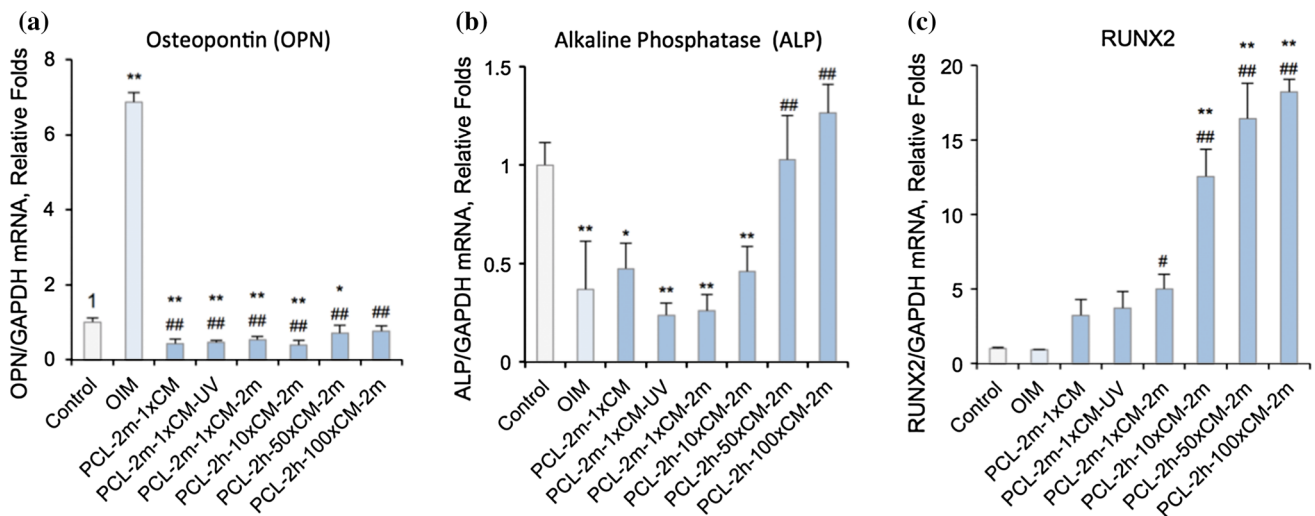


Figure 6 Osteogenic marker expression for MSCs cultured on modified PCL, as a function of processing conditions after 7 days. PCL treatment with increased concentrations of CM resulted in higher ALP and Runx2 expression, while OPN was largely unaffected. Asterisk and double asterisk stand for significant

difference between samples and the control with $p < 0.05$ and 0.01 , respectively. # and ## stand for significant difference between samples and the positive control OIM with $p < 0.05$ and 0.01 , respectively.

MTT values than control in cells differentiated on PCL films modified using $10\times$ (PCL-2 h- $10\times$ CM) and $50\times$ of CM (PCL-2 h- $50\times$ CM-2 m). These two conditions can induce significantly higher cell

activities throughout 7 days of differentiation. However, in the sample PCL-2 m- $1\times$ CM, the MTT value is lower than the control throughout the 7-day differentiation period.

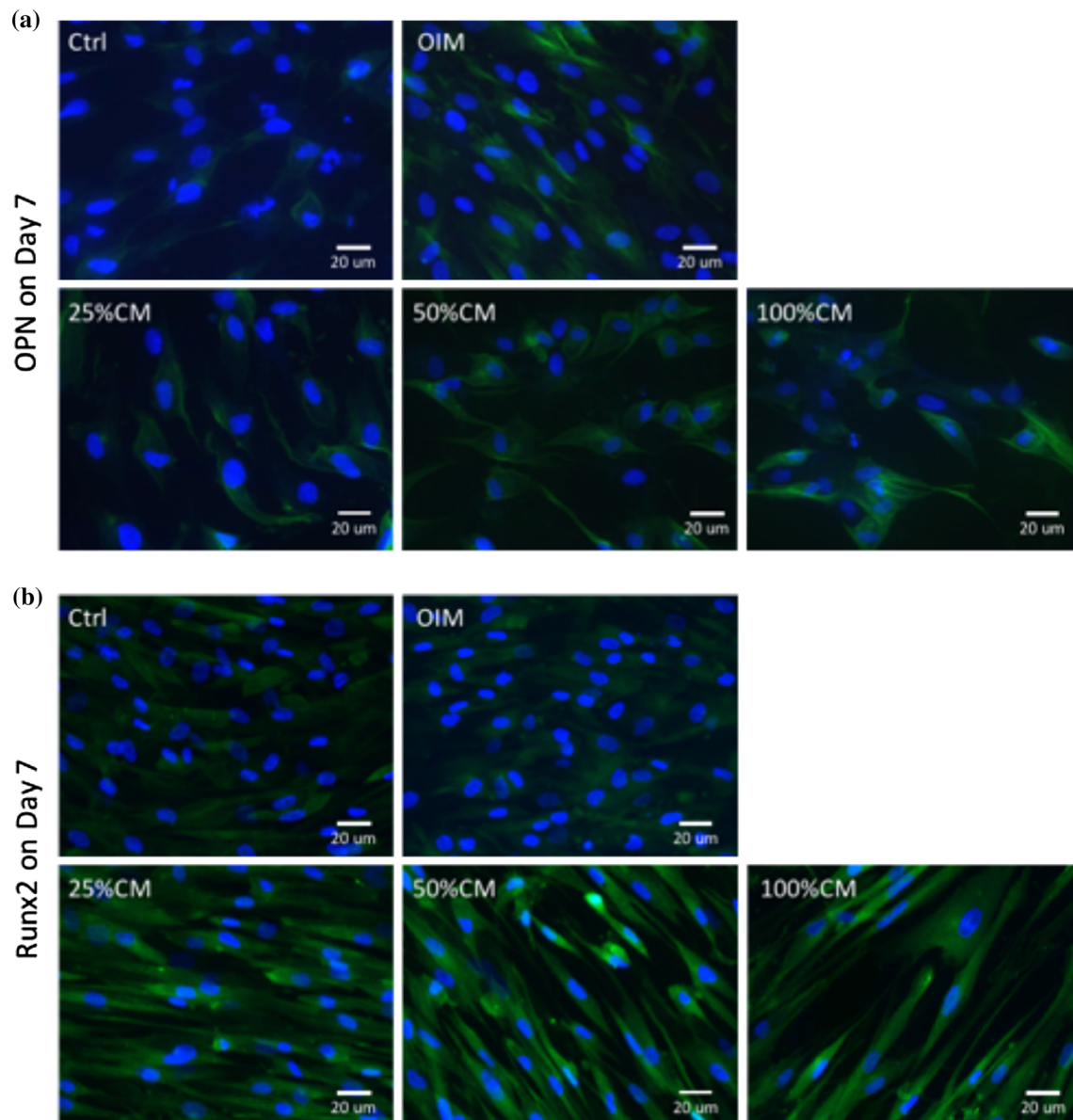


Figure 7 Immunostaining of MSCs for OPN (*panel a*) and Runx2 (*panel b*) cultured under different media. The images were taken after culturing for 7 days. The scale bar represents 20 µm.

Evaluation of mRNA expression of osteogenic markers

We used real-time PCR analysis to detect osteopontin (OPN), Runx2, and alkaline phosphatase (ALP) for osteogenic differentiation. We verified both the effects of different CM concentrations (Fig. 5) and different CM-modified PCL films on the differentiating MSCs (Fig. 6). As shown in Fig. 5, we did not see any OPN mRNA expression in MSCs after 3 days. However, after 7 days we detected mRNA expression of OPN and found that it was dramatically increased in MSCs

cultivated with OIM, and different concentrations of MG63 CM (Fig. 5a). We found that OPN had a higher mRNA expression in MG63 CM than in OIM. Similarly, the mRNA expression of ALP (Fig. 5b) was upregulated in all three CM concentrations after seven days of culturing. For Runx2, only the group with 100% CM showed significantly higher expression. These results demonstrate that, in comparison to OIM, the MG63 CM can increase expressions of osteogenic markers in MSCs, and sometimes significantly so.

We then studied whether our CM-modified PCL scaffolds could promote osteogenic marker

expression (Fig. 6). The unmodified PCL scaffold (Fig. 6a) had the highest OPN mRNA expression. Among the modified PCL scaffolds, PCL treatment with higher CM concentrations had the best effect, both on OPN mRNA expression and ALP mRNA expression (Fig. 6b). The Runx2 mRNA expression showed a significant increase with treatment concentration (Fig. 6c). The results show that different PCL surface modifications can still induce expression levels of osteogenic markers, and it reveals possible dependencies of the growth on the concentration during PCL treatment, and the discussion section will address in more detail the significance of the concentration dependencies in Figs. 5 and 6. The osteogenic expression profiles of cells differentiated on CM-modified PCL groups were different from the cells treated with conditioned medium, however.

Immunostaining of MSCs cultured in MG63-conditioned medium

Immunostaining for OPN and Runx2 was performed on MSCs at the end of day 7 of the differentiation culture (Fig. 7). For MSCs cultured in OIM and 25% MG63 CM, we found a weaker immunostaining for OPN (Fig. 7a). However, MSCs cultured in 50 and 100% MG63 CM showed a high expression of OPN. In addition, we found that Runx2 protein expression was dramatically increased in MSCs cultured with 25, 50, and 100% MG63 CM (Fig. 7b). These data show that MG63 CM increases the protein expression of the osteogenic markers OPN and Runx2, which are in agree with the mRNA expression profile of the cells.

Discussion

In this study, we first analyzed the effects of MSC growth in different MG63 CM compared to OIM (Fig. 4b). It is interesting to find that MG63 CM has a higher capacity for osteogenic differentiation than OIM (Fig. 5). We further treated the PCL films with MG63 CM to produce a bioactive surface that can gradually release proteins (Fig. 3), which were proven to induce MSC differentiation (Fig. 6). This approach has never been reported, according to the authors' knowledge.

MSCs are known to differentiate into osteogenic cell lineages in OIM. However, the OIM method

requires at least fourteen days, frequent media refreshing, and a larger number of different reagents. The alternative method we have demonstrated here can shorten the differentiation time, without the changes in required media which the OIM requires. Previous studies have reported that BMP is present in abundance in MG63 CM [17] and that it is capable of facilitating cell differentiation [20]. BMP signaling is considered to be one of the central signaling pathways involved in osteogenic differentiation and regulation of bone formation [29]. The CM contains further growth factors and bioactive molecules that help induce cell proliferation and differentiation. Thus, for cells cultured in MG63 CM, we found that osteogenic markers can be detected as early as on the day 7 (Figs. 5, 6), accelerating the stimulation compared to OIM. Similarly, a study that focused on CM from the MG63 osteosarcoma cell line reported that CM is able to induce a significantly higher rate of osteoclastogenesis.

To enable PCL scaffold modification, we treated the PCL with CO₂ plasma and studied the impact of the duration of plasma exposure, using 2, 30 m, and 2 h (Figs. 2, 3, 6). The CO₂ plasma treatment modifies the functional groups of the surface, and also results in cutting of PCL polymer backbones, which lowers the molecular weight of PCL near the surface. Therefore, proteins conjugated to lower molecular weight PCL can be more easily released from PCL surfaces through the hydrolysis process. For all durations, the treatment yielded a burst release of protein from modified PCL scaffolds on the first day, but we found that the modified PCL scaffold was stable after the 2 h treatment (Fig. 3). On the other hand, a short-term CO₂ plasma treatment limits the conjugation of bioactive molecular onto the PCL scaffolds, while for a longer CO₂ plasmas treatment, the availability of carboxylic acid group is increased. This in turn allows a higher degree of conjugation of bioactive molecules from the MG63 CM onto the scaffold surfaces. We suspected there is protein adsorption for why proteins are only released initially but not over the entire 7 days period.

For MSCs, cell proliferation occurs prior to cell differentiation. MTT values are often used to measure the number of proliferated cells. Based on the higher expression of osteogenic markers that we saw with MG63 CM in comparison to OIM, we initially expected that MG63 CM would also show higher MTT values compared to OIM. Surprisingly, we

found the inverse: OIM showed a higher MTT value than MG63 CM (Fig. 4). However, while OIM yielded a higher number of proliferated cells, not all these cells necessarily translate into differentiated cells. On the other hand, the lower yield of proliferated cells for MG63 CM had a higher rate of proliferated cells that translated into osteogenic cells. Therefore, MG63 CM promotes more efficient and targeted cell differentiation when it comes to stimulating osteogenic lineage from MSCs. Additionally, visual monitoring did not find a noticeable amount of cell death (data not shown), and we conclude that MG63 CM is not a selecting medium that kills cells that have not differentiated into osteoblastic lineages.

Our data showed that MG63 CM upregulated mRNA expression in MSCs of Runx2 and OPN, but not of ALP (Fig. 5). The effects of BMP to differentiate MSCs into osteoblast have been reported in several studies. Previous studies showed that rat bone marrow MSCs when cultured with BMP-2 induced osteocalcin (OCN) expression and alkaline phosphatase (ALP) activity [30]. BMP was also found to upregulate Runx-2 expression under certain conditions [27, 28]. Because BMP is abundant in MG63 CM, we believe that the MG63 CM treatment-induced osteogenic marker Runx2 expression occurs through the BMP signaling pathway. Several studies have indicated that Runx2 can stimulate many genes, including OCN, collagen I, collagenase III, and ALP [23–26]. The Runx2 gene is more specifically upregulated in early osteoblast progenitors [23]. In this study, the Runx2 mRNA expressions in MG63 CM are much higher than in OIM, suggesting that MG63 CM could facilitate Runx2 to lead the formation of pre-osteoblasts. In addition, Runx2 has been shown to induce ALP activity [26]. Our data showed these results occur on day seven (Fig. 5). Therefore, we expect that ALP mRNA expression increased over time. When the protein conjugation occurs on a surface, the protein conformation may change and thus expose different bioactive motifs depending on the extent of the conformational changes. Therefore, we may question whether protein conjugation onto a surface changes the conformation and thus affects bioactivity. Since our data indicated that a higher concentration of MG63 CM yields larger amounts of osteogenic markers, we conclude that the functionality of proteins are not reduced by conjugating them onto the PCL surface.

Conclusion

In this study we have found a differentiation strategy to differentiate MSCs into osteogenic cells using MG63-conditioned medium. This approach can be further developed as “bioactive surface modification” by conjugating the molecules from other CM onto the PCL films. In contrast to conventional differentiation methods, our approach holds the promise of creating a simpler and faster method, with equivalent results, but one that can be automated and scaled up resulting in cost effective manufacturing processes. This would present a significant advance, because MSC users would be able to avoid the need to manually perform CM collection, and could instead benefit from a standardized and consistent off-the-shelf solution.

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