Polyelectrolyte multilayer (PEM) film can modify the surface properties of materials to improve cellular responses. In this study poly(diallyldimethylammonium chloride) (PDADMAC), poly(sodium 4-styrene sulfonate) (PSS) and poly(4-styrenesulfonic acid-co-maleic acid) sodium salt (PSS-co-MA) were assembled into PEM ([PDADMAC/PSS]/PDADMAC+PSS-co-MA) film on glass surfaces and its ability on affecting osteoblast functions were examined. PSS-co-MA film showed an increase roughness and more wettable surface as compared to glass. When the osteoblast cell line, MC3T3-E1, was seeded on the surfaces, no differences were observed in cell attachment or spreading on either PSS-co-MA film or glass at 4-16 hours. However, increases in alkaline phosphatase activity (day-5 and 7) and the expression of osteocalcin mRNA/protein at day-13 were observed. Cells cultured on PSS-co-MA film developed a faster rate of calcium deposition at day-15 compared to control. In conclusion, PSS-co-MA film enhanced osteoblast differentiation and could be used to promote mineralization and improve osseointegration for dental implants.

**Keywords:** Poly(4-styrenesulfonic acid-co-maleic acid) sodium salt, Polyelectrolyte multilayer films, Osteogenic differentiation, Mineralization
Poly(diallyldimethylammonium chloride) (PDADMAC) is a strong cationic polyelectrolyte, containing numerous positive charges along its backbone chain. In contrast, PSS, a strong anionic polyelectrolyte, possesses a number of negative charges along its backbone chain. Due to their strong ionic charges, both PDADMAC and PSS have been used as the polyelectrolytes for PEM preparation\(^{22-25}\). Since the hydrophobic ring structure of PDADMAC polycations is stiff and consequently difficult to rotate both in water and in air, the outer layer containing the quaternary ammonium end groups will stay hydrophilic both in water and in air. The hydrophilic property of the polycations will help the addition of the next PSS layer\(^{26}\). Another advantage of using strong polyelectrolytes is their ionic charges are largely independent of the pH condition\(^{27}\). These characteristics support the advantage of using PDADMAC and PSS for PEM fabrication.

Poly(4-styrenesulfonic acid-co-maleic acid) sodium salt (PSS-co-MA) was selected for coating the final layer of PEM. PSS-co-MA is a copolymer of PSS and maleic acid. This copolymer contains both the strong sulfonate group in PSS and the weak carboxylic pendent group from maleic acid segments. The strongly charged group can generate electrostatic linkages thereby enhancing the film stability. Meanwhile, the weakly charged groups of maleic acid provide flexibility to the multilayer properties due to their ability to respond to external pH changes. For example, at high pH, the carboxylic acid group in maleic acid could be converted into a carboxylate group and become ionized, with the PSS-co-MA becoming an anionic polyelectrolyte\(^{28}\). Due to this property, PSS-co-MA has been used as a cation-exchange membrane, since the maleic acid has two ion-exchangeable sites and exhibits lower water uptake than sulfonic acid\(^{29}\). However, it was never been applied in order to influence osteoblast behavior.

The objective of this study was to fabricate PEM films using PSS-co-MA, PDADMAC and PSS. The characteristics of the surface modified films and their effects on osteoblast cells were investigated.

MATERIALS AND METHODS

Fabrication of polyelectrolyte multilayer films

Glass cover slips (12 mm round or 22×22 mm\(^2\) square)
were pretreated with freshly prepared piranha solution, a 30:70 v/v mixture of 40% hydrogen peroxide and concentrated sulfuric acid, for 10 minutes followed by immersing in 1% ammonia solution for another 10 minutes. The pretreated glass surfaces were thoroughly rinsed three times with distilled water and air dried.

Polyelectrolyte multilayer films were constructed by forming 9 layers of PDADMAC and PSS with a final layer of PSS-co-MA ([PDADMAC/PSS]/PDADMAC+PSS-co-MA) on glass surfaces. Briefly, the pretreated cover slips were alternately immersed in 10 mM PDADMAC in 0.1 M NaCl or 10 mM PSS in 0.1 M NaCl for 5 minutes, respectively, with intermediate triple rinses with distilled water until the ninth layer was formed. For the final layer, the glasses were immersed in 10 mM PSS-co-MA (pH 10) in 0.1 M NaCl for 30 minutes, rinsed three times with distilled water (pH 10) and air dried resulting in a polyanionic surface. PDADMAC, PSS and PSS-co-MA were purchased from Aldrich (St. Louis, MO, USA). Chemical structure of each polyelectrolyte used was shown in Fig. 1a.

UV-vis spectroscopy

PEM films were assembled on fused quartz plates (2 mm thick, 1 inch diameter, GM Associates, Oakland, CA, USA) which are transparent to the UV-visible spectrum. The plates were pretreated with piranha solution, 1% ammonia and rinsed with distilled water. UV-vis absorption spectra were recorded using a UV-vis Spectrophotometer (SPECORD® S100, Analytik Jena AG, Jena, Germany). This method was used to measure the thickness of PDADMAC/PSS multilayer and PSS-co-MA adsorption onto PEM. PEM buildup can be monitored with this method since the amount of polymer deposited is proportional to the absorbance according to Beer's law: A=ε.b.C, where A is the absorbance, ε is the molar absorptivity (Lmol⁻¹ cm⁻¹), b is the absorbing medium pathlength (cm) and C is the concentration (mol⁻¹).

Surface characterization analysis

Surface roughness was measured by atomic force spectroscopy (AFM; Nanoscope IV, Multimode, Veeco, Santa Barbara, CA, USA). Average surface roughness (R_a), the root mean square (rms) roughness (R_q) and thickness of the PSS-co-MA film were calculated from measurement of three independent samples. Surface morphology of the specimens was also examined using a scanning electron microscope (SEM; JSM-5410LV, JEOL, Tokyo, Japan) and atomic force microscope (Nanoscope IV, Multimode, Veeco, Santa Barbara, CA, USA).

Hydrophilicity

Static contact angle measurement was performed using a contact angle meter (DSA 10, Krüss, Hamburg, Germany) at room temperature to determine the existence of hydrophilic sites on both glass surfaces and PSS-co-MA-coated PEM surfaces. The samples were kept in the ambient environment for 15 minutes to set as a standard for contact angle measurement in this work. To perform this measurement, a 10 µL droplet of de-ionized water was dropped vertically on the specimen surface without any physical contacts with a micro-syringe onto the film surface. The contact angles were measured ten times and then averaged.

Cell culture

MC3T3-E1 cells (ATCC CRL-2593), an immortalized cell line derived from mouse calvarium tissue, were maintained in minimum essential medium (HyQ® MEM/EBSS, Hycone, Logan, Utah, USA) supplemented with 10% fetal bovine serum (FBS, ICP biologicals, Henderson, Auckland, New Zealand), 2 mM L-glutamine, 100 unit mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin and 0.25 µg mL⁻¹ amphotericin B (Gibco, Grand Island, NY, USA) under standard condition (at 37°C in 5% CO₂). Cell from passage 18 to 22 were used in the experiments. For MTT assay, SEM, alkaline phosphatase (ALP) activity, quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and osteocalcin (OC) protein analysis, cells were seeded on glass cover slips (diameter 12 mm) in 24 well plates at a density of 40,000 cells per well. For Alizarin red staining, cells were seeded on 22×22 mm² glass cover slips in 6 well plates at a density of 200,000 cells per well. The cover slips were sterilized with 70% ethanol, rinsed with culture medium and air dried before use. The medium was changed every other day.

Scanning electron microscopy (SEM)

For SEM analysis, MC3T3-E1 cells were cultured on uncoated and PSS-co-MA-coated PEM glass for 4 and 16 hours. Cells were rinsed twice with phosphate buffered saline (PBS) and fixed with 3% glutaraldehyde solution (Fluka, Milwaukee, WI, USA) dilute with 0.1 M PBS 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%), and then critical point dried with 100% hexamethyldisilazane (HMDS; Fluka, Steinheim, Germany) for 5 minutes. Gold was sputter-coated on the surface and the samples were examined using a scanning electron microscope (JSM-5410LV, JEOL, Tokyo, Japan).

MTT assay

MC3T3-E1 cells were seeded on glass cover slips (diameter 12 mm) in 24 well plates at a density of 40,000 cells per well. Cell number was determined by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-dipheyl tetrazolium bromide) assay. MTT (USB Corporation, Cleveland, OH, USA) solution of 5 mg mL⁻¹ was prepared by dissolving MTT in 10% serum culture medium without phenol red. At the end of culture periods (4 and 16 hours), cells were washed with PBS prior to the addition of MTT solution into each well and incubated for 15 minutes at 37°C. At the end of the assay, the blue formazan reaction product was dissolved by 1 mL of glycine buffer (pH 10) (125 µL/well) and dimethyl sulfoxide (DMSO; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) (900 µL/well). The optical density of this colored solution in each well,
representing the number of viable cells, was measured using a spectrophotometer (Thermospectronic Genesis10 UV-vis, Madison, WI, USA) at a wavelength of 570 nm. Cell numbers were determined according to the standard curve of relative known cell number.

**Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis**
MC3T3-E1 cells were seeded on tissue culture plate, glass cover slip and PSS-co-MA coated glass and cultured for 13 days. Expressions of type I collagen (Col I), osteopontin (OPN), bone sialoprotein (BSP), and osteocalcin (OC) messenger RNA (mRNA) were assessed using qRT-PCR. The MC3T3-E1 cells were detached from samples using 1 mL of 0.2% EDTA (ethylenediaminetetraacetic acid; Sigma, St. Louis, MO, USA) in PBS. A cell pellet was obtained by centrifugation at 12,000×g (14,000 rpm) for 10 minutes and the RNA was extracted with 1 mL of TRIzol Isolation Reagent (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer protocol. RNA yields were evaluated with a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA) based on the absorbance ratio at 260/280 nm. First strand DNA was reverse transcribed from 1 µg of total RNA using reverse transcriptase enzyme (ImProm-II Reserve Transcription System, Promega, Madison, WI, USA).

qPCR was performed using the LightCycler 480 (Roche, Mannheim, Germany) and LightCycler® SYBR Green I Master (Roche, Mannheim, Germany) in a 10 µL reaction volume under the following cycling conditions: 95°C, 5 min, followed by 40 cycles of 95°C for 10 s; 60°C for 10 s; 72°C for 25 s. PCR oligonucleotide sequences of the primers are followed: Col I sense 5’GGT GCC CCC GGT CTT CAG3’, antisense5’AGG GCC AGG GGG TCC AGC ATT TC3’, OPN sense 5’CCA ACG GCC GAG GTG ATA3’, antisense 5’CAG GCT GGC TTT GGA ACT TG3’, BSP sense 5’TGT CTG CTG AAA CCC GTP C3’, antisense 5’GGG GTG TTT AAG TAC CGG C3’, OC sense 5’CTT GGG TTC TGA CTG GTG GT3’, antisense 5’AGG GAG GAT CAA GTC CCGC3’ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense 5’ACT TTT TCA AGC TCA TTC C3’ and antisense 5’TGC GAA GAA CTT TAT TGA TG3’. The primer was designed from the sequence in GenBank database (NM_007742.3, NM_009263.1, NM_008318.1, NM_001032298.2 and XM_001476723.1 for Col I, OPN, BSP, OC and GAPDH, respectively). All samples were performed in triplicates and the house keeping gene, GAPDH, was used as a reference control. The calculations of average Cp values and resulting expression ratios for each gene were performed using the Roche LightCycler 480 software version 1.5 (Roche, Mannheim, Germany).

**Alkaline phosphatase activity (ALP activity)**
Cells were rinsed with PBS and scraped in alkaline lysis buffer (10 mM Tris-HCl, 2 mM MgCl₂, 0.1% Triton-X100, pH 10) (100 µL/well). A half volume of each sample was mixed with ALP substrate solution containing 2mg/mL p-nitrophenyl phosphate (PNPP; Zymed, Invitrogen, Carlsbad, CA, USA) in 0.1 M 2-amino-2-methyl-1-propanol, 2mM MgCl₂, pH 10.5 for 10 minutes at 37°C. The reaction was stopped by the addition of 0.9 mL/well of 0.1 M NaOH, and the absorbance was read at 410 nm. The other half volume of each sample was used for protein quantification using a bicinchoninic acid protein assay (BCA™, Thermo Scientific, Rockford, IL, USA). The absorbance for the protein assay was read at 562 nm. The amount of ALP was calculated as nanomolar of p-nitrophenyl/µg protein/min.

**Osteocalcin analysis using enzyme-linked immunosorbent assay (ELISA)**
MC3T3-E1 cells were seeded on tissue culture plate (TCP), glass cover slip and PSS-co-MA coated glass and incubated for 13 days, using mouse osteocalcin ELA kit (Biomedical Technologies Inc., Stoughton, MA, USA). The culture medium was changed 1 day prior to sample collection. The supernatants of cell seeded on each sample were used to analyze osteocalcin protein according to manufacturer's instruction.

**Alizarin red-S staining**
Calcium deposition was quantified by Alizarin red-S staining (Alizarin Red S certified, Sigma, St. Louis, MO, USA). MC3T3-E1 cells were cultured overnight on uncoated glass or PSS-co-MA-coated PEM and the uncoated glass cover slips. Data were presented as the mean±SD.

**Statistical analysis**
Data were analyzed using statistical software (SPSS® 15.0 for Windows, SPSS, Chicago, IL, USA). A two-tailed students' t-test was used to compare results from the PSS-co-MA-coated PEM and the uncoated glass cover slips. Data were presented as the mean±SD. p-values <0.05 were considered as significant.

**RESULTS**

**Formation of (PDADMAC/PSS)₄/PDADMAC+PSS-co-MA multilayer**
The presence and growth of multilayers of PDADMAC, PSS and PSS-co-MA solutions in 0.1 M NaCl was
monitored using UV-vis spectroscopy. The chemical structure of PDADMAC, PSS and PSS-co-MA was shown in Fig.1a. PSS and PSS-co-MA have an absorption peak at 226 nm due to the sulfonate phenyl ring in the PSS moiety, while PDADMAC showed no absorption in the UV-vis region. For the UV-vis measurement, the PSS absorption was monitored as a function of the layer number to obtain information on the absorbed amount of PSS at 226 nm. The absorbance of PSS was found to increase with increasing numbers of layers (Fig. 1b). The PSS-co-MA absorbance increased rapidly after 30 seconds of dipping and became constant after 2 minutes (Fig. 1c). These results indicated the success of PEM film construction with polyelectrolyte solutions.

Hydrophilicity

The wettability of the surface was measured by dropping a drop of water onto the PSS-co-MA coated surfaces and uncoated glass. Results showed the water-drop spread more on PSS-co-MA coated surfaces than on uncoated glass as shown by the side view images (Fig. 2a). The contact angle of a water-drop on PSS-co-MA coated surfaces was significantly lower (18.56°) than on uncoated glass (31.44°) (Fig. 2b), indicating PSS-co-MA coated surfaces possessed a greater wettability than the glass surfaces.

Surface characterization analysis

Results from AFM analysis revealed the significant difference between the glass and PSS-co-MA coated surfaces as showed in Table 1 ($p<0.01$ for both $R_a$ and $R_q$). The $R_a$ of glass and PSS-co-MA coated surfaces were 0.350±0.02 nm and 1.421±0.04 nm, respectively. The $R_q$ values of glass and PSS-co-MA coated surfaces were 0.421±0.01 nm and 1.857±0.03 nm, respectively. In addition, AFM results also revealed that the average thickness of PSS-co-MA PEM film was 9.088±1.37 nm.

The 3-dimensional images from AFM (Fig. 3a1, 3a2) showed significant difference in surface topography between the glass and PSS-co-MA coated surfaces. PSS-co-MA coated films revealed the uniform and homogeneity. However, SEM examination showed that there was no significant difference in surface topography between the glass and PSS-co-MA coated surfaces (Fig. 3a3, 3a4). This result demonstrated that SEM analysis failed to represent true nanoscale feature of PSS-co-MA PEM surfaces.

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**Fig. 2** The wettability of glass and PSS-co-MA PEM surfaces

Photograph of water dropped on glass and PSS-co-MA coated surface (a). The water contact angle of glass and PSS-co-MA coated surfaces (b). Data was shown as the mean±SD. * Statistically significant, $p<0.05$

**Table 1** Surface roughness determination of Glass surface and PSS-co-MA PEM surface

<table>
<thead>
<tr>
<th>Materials</th>
<th>$R_a$ (nm)</th>
<th>$R_q$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass surface</td>
<td>0.350 (0.02)</td>
<td>0.421 (0.01)</td>
</tr>
<tr>
<td>PSS-co-MA PEM surface</td>
<td>1.421 (0.04)*</td>
<td>1.857 (0.03)*</td>
</tr>
</tbody>
</table>

Data were shown as the mean (SD). * Statistically significant, $p<0.05$
Fig. 3  Surface characterization and morphology of MC3T3-E1 cells on glass and PSS-co-MA PEM film
Surface characteristics (a) of the glasses cover slip surface (a1, a3) and PSS-co-MA PEM coated surfaces (a2, a4) analyzed by atomic force microscopy (a1, a2) and scanning electron microscopy (a3, a4). Adhesion of MC3T3-E1 cells on glass (b1, b3) and PSS-co-MA PEM surfaces (b2, b4) at 4 (b1, b2) and 16 hours (b3, b4).
coated films.

Cell morphology
The morphology of MC3T3-E1 on both surfaces was analyzed after seeding for 4 and 16 hours. Cells on both surfaces appeared to be well spread at both time points. At 4 hours, cells appeared flattened and started to form contacts with the adjacent cells (Fig. 3b1, 3b2). At 16 hours, a tightly packed cell layer was observed (Fig. 3b3, 3b4).

Cell adhesion
The number of the cells increased during the culture period (from 4–16 hours) on both surfaces. Although the number of cells was slightly less on PSS-co-MA PEM compared to the uncoated glass, no statistically significant differences between the two groups were detected (\( p=0.226 \) for 4 hours and \( p=0.159 \) for 16 hours) as shown in Fig 4.

ALP activity
The ALP activity, an early marker of osteoblast
differentiation, of MC3T3-E1 cultured on both surfaces was monitored on days 3, 5 and 7 after seeding (Fig. 5). The ALP activity of cells seeded on PSS-co-MA PEM was higher than that on the uncoated glass at day-5 with a significant difference at day-7 ($p=0.027$).

Osteoblastic gene expression
The differentiation of osteoblast was further indicated by the expression of Col I, OPN, BSP, and OC at day-13 (Fig. 6a). Results from qRT-PCR revealed the 6-fold increase on the expression of BSP and OC in cell cultured on PSS-co-MA coated surface compared to glass cover slip control, while the expression of Col I and OPN were slightly increased (2 fold). To confirm the increase in OC expression, ELISA analysis of OC was also performed at day-13. Significantly increased of OC synthesis was observed in PSS-co-MA coated films compared to glass surfaces (Fig. 6b).

In vitro calcification
Cells were cultured in the presence of ascorbic acid and β-glycerophosphate for 15 days. The cultures were fixed and stained with Alizarin red-S to evaluate the presence of *in vitro* calcification. Macroscopically and microscopically observations revealed more reddish deposition in cultures on PSS-co-MA coated surfaces than on glass surfaces (Fig. 7a). Figure 7b showed a higher magnification of cells in Fig. 7a (on PSS-co-MA coated surface).

The amount of calcium deposition on each surface was quantified calorimetrically using 10% cetylpyridinium chloride monohydrate. The result showed that the amount of calcium deposition on PSS-co-MA coated surfaces was significantly greater than the control groups ($p=0.003$) (Fig. 7c).

**DISCUSSION**
This is the first study demonstrating the ability of PSS-co-MA coated PEM surfaces, used as a novel nanoscale surface modification, to affect osteoblast function and differentiation. Results indicated that the coated PSS-co-MA PEM glass surfaces could enhance osteoblast differentiation as measured by osteoblastic
gene expression, ALP activity, OC protein synthesis and \textit{in vitro} calcification.

Generally, major phases involved in osteoblast differentiation include proliferation period, extracellular matrix deposition and maturation, and subsequently mineralization\(^{30}\). Various genes were reported to be regulated or highly expressed in certain stage of osteoblast differentiation and these genes could be used as the specific markers at each stage of differentiation. Col I and ALP are highly expressed near the end of the proliferative period and during the period of extracellular matrix deposition and maturation, while OPN, BSP and OC are highly expressed at or near the time of mineralization\(^{30}\). Col I is the most abundant protein in bone matrix serves as a template for mineralization\(^{32}\). Moreover, Col I is an essential matrix protein that plays a fundamental role in the maintenance of osteoblastic phenotype making the matrix competent for mineralization\(^{33}\). ALP, a membrane bound enzyme, is abundantly expressed in early stages of bone formation and has been considered as an early marker of osteoblast differentiation. Increased ALP levels correlated with increased bone formation histomorphometrically\(^{34}\).

OPN is a phosphorylated glycoprotein which contains a string of polyaspartic acid residues as well as a arginine-glycine-aspartate (RGD) sequence. OPN is believed to facilitate the attachment of osteoblasts and osteoclasts to the extracellular matrix, allowing them to perform their respective functions during osteogenesis\(^{35}\). BSP is a highly glycosylated and sulphated phosphoprotein that is almost exclusively found in mineralized connective tissues such as bone and cementum. BSP contains large stretches of poly(glutamic acids) as well as the RGD sequence at its carboxyl terminus with the ability to bind hydroxyapatite and cell-surface integrins and acts as a nucletor of the initial apatite crystal\(^{32, 36}\). OC or \(\gamma\)-carboxyglutamic acid or Gla protein, an abundant Ca\(^{2+}\) binding protein commonly found in the organic matrix of mineralized tissues such as bone, dentin, and cementum\(^{37}\), is highly expressed at or near the time of mineralization and has been considered as the late-stage marker of osteoblast differentiation. In this study, it is well demonstrated that the PSS-co-MA films can induce an increase in ALP activity, expression of osteogenic marker genes including Col I, OPN, BSP, and OC and accelerate the \textit{in vitro} calcification. These suggested the role of PSS-co-MA PEM films in osteoblast differentiation and its possible clinical application in using with endosseous implant.

In this study, PDADMAC and PSS were employed for PEM process. As it was previously reported that the biological responses to PEM film depended on the outer layer of film\(^{38, 39}\), PSS-co-MA was used as a final depositing layer of the film to evaluate its effect on osteoblast behavior. To prove of principle that PSS-co-MA influences osteoblast behaviors, (PDADMAC/PSS)/PDADMAC+PSS-co-MA film was fabricated on glass surface. It has been proposed that the influence of PEM film on cellular behaviors depended on the coated surface regardless of the substrate used. Elbert et al. demonstrated that when bioactive surface, \textit{i.e.} TCP, gelatin adsorbed TCP or fibroblast extracellular matrix, was coated with polylysine/alginie PEM film, the decrease of cell spreading was observed in all modified surfaces\(^{40}\), suggesting the momentous influence of polylysine/alginie PEM coated surface, not substrate materials, on cellular response. In addition, it has also been reported that fibroblast cells response differently depended on the type and nature of coated PEM film, supporting the crucial role of material’s surface properties on biological interaction\(^{41}\). Moreover, we currently are under investigations of similar coating strategy on titanium surface to examine the biological responses both \textit{in vitro} and \textit{in vivo}. From the preliminary results, the similar trends of osteoblast response were observed on (PDADMAC/PSS)/PDADMAC+PSS-co-MA coated titanium surface compared to those on glass surface. These may lead to potential application of (PDADMAC/PSS)/PDADMAC+PSS-co-MA coated film for endosseous titanium implants.

In this present study, AFM analysis showed the existence of uniform and homogeneous polyelectrolyte deposition on the glass surface. In addition, the thickness of PSS-co-MA film could be measured, confirming the presence of PSS-co-MA film on glass surface. It is well established that surface roughness is one of the important parameters that could influence cellular behavior. It has been reported that roughness in a nanoscale range could affect osteoblast adhesion and differentiation\(^{42}\). However, the effects of surface roughness on osteoblast behavior were still controversial depending upon the degree of the roughness. In present study, the roughness of PSS-co-MA surface was significantly higher than glass surfaces. However, when glass surfaces were coated with only PSS-co-MA, which also produce the similar roughness as of the PEM, no effect on osteoblast behavior as compared to the control was observed (data not shown). Therefore, the increase surface roughness in this study may not directly correlate to osteoblast differentiation.

Furthermore, PSS-co-MA coated surfaces had better wettability as compared to control uncoated glass. It is well documented that hydrophilic/hydrophobic balance of a given surface is a major parameter affecting cellular behavior at the cell-materials interface. However, effects of surface wettability on cellular behavior are reported to be inconsistent and controversial. For example, it had been shown that hydrophobic materials effected greater cell attachment than hydrophilic materials\(^{43}\). In contrast, there were studies demonstrating positive effects of hydrophilic surfaces on cell adhesion for various cell types whereas moderately hydrophobic surfaces often inhibited cell-material interaction\(^{44, 46}\).

Despite the better hydrophilicity, the number of cells adhering on PSS-co-MA coated films was comparable to glass surfaces, suggesting hydrophilicity may not be the sole factor involved in cell attachment. The result of this study are in agreement with a report from Faucheux and coworkers\(^{46}\), comparing the attachment of human fibroblasts on various modified surfaces, using different
terminating functional groups. Weak interaction was observed when cells were grown on self-assembling monolayers (SAMs) terminating with methyl (CH₃) groups, which is considered a hydrophobic surface. In contrast, strong cell attachment, spreading, and growth were found in cells cultured on moderate hydrophilic COOH terminated SAMs. Therefore, cell attachment and spreading might also depend on the different terminal functional groups on the material's surface.

During the process of surface modification, it is extremely difficult to control one factor such as surface composition without changing others such as surface topography, roughness and wettability, preventing the identification of the genuine effect of one factor. Modifying titanium implant surfaces can alter their roughness, wettability and chemical composition resulting in optimizing interactions at the biosystem-material interface. Surface wettability, is not solely determined by the micro- or nanostructure of surfaces, but also by the chemical composition, i.e. by the presence of hydroxyl, carboxyl, amine and other organic or inorganic chemical groups.

In terms of surface chemistry, studies suggested that nature and chemical composition of PEM films could affect adhesion and proliferation of cells cultured on the films. Various cell types such as fibroblasts, osteoblast-like cells (SaOS-2, MC3T3-E1), human periodontal ligament (PDL) cells, endothelial cells and various hepatocytes (human hepatocellular carcinoma cells, adult rat hepatocytes and human fetal hepatoblasts) have been studied. For example, Tryoen-TÓth et al. suggested that a good cell adhesion, proliferation and stability of osteoblast phenotype of PSS- and PGA-ending films for PDL cells and of PSS-, PGA-, and PLL-terminating films for SaOS-2. On the other hand, the PEI-terminating layers were cytotoxic for both cells. Furthermore, results of Chua et al. showed that Chi-graft titanium substrates were better support MC3T3E-1 cell adhesion than hyaluronic acid (HA)-graft titanium and HA/Chi PEM functionalized titanium. Moreover, the immobilization of RGD peptide on the HA/Chi PEM functionalized titanium had a significant effect on osteoblast proliferation and ALP activity while retaining high antibacterial efficacy. These results indicated the importance of chemical composition on the modified surfaces on cell behavior.

Evidences indicated that differentiation of osteoblasts on the material surface depend on material surface chemistry and topography. Previously we and others showed that different type of titanium (commercially pure titanium and titanium alloys) showed different effect on function and differentiation of osteoblast. It has been proposed that the differences may due to the surface chemistry. Report from Scopelliti et al. showed that surface nanostructur effect cell behavior through the ability and type of protein adsorption. They suggested that different types of protein that aggregated on the materials surface provided the environment for cell adhesion and subsequently differentiation. Furthermore, the works of Keselowsky et al. revealed that different form of chemical groups adsorbed on the surface affected the cellular behavior differently. These data supported the importance of surface chemistry compatibility on protein adsorption, attachment and differentiation of osteoblast on the implant materials.

The effect of surface chemistry on cell-material interactions might be considered as secondary effects. The type and amount of proteins adsorbed on the surface may act as a primary factor governing the cell-surface interactions. This hypothesis is in agreement with the work reported by Ma et al., who showed the hydrophilic and hydrophobic surface did not directly govern cell attachment. Rather, the ability of moderate wettable surfaces in adsorbing a proper amount of protein is the key for improving cell-materials interactions. In this study, the upper surface of PEM contained maleic acid. Maleic has been used in several studies to prepare the materials surface for protein adsorption such as fibronectin (FN). Renner et al. indicated that maleic anhydride coating was suitable for FN adsorption. FN is a multifunctional glycoprotein that plays an important role in osteoblast differentiation. Moursi et al. used a polyclonal anti-FN antibody to study the role of FN in the progressive differentiation of osteoblast. They found that osteoblast interactions with the central cell-binding domain of FN (integrin-mediated mechanism) are required for in vitro nodule formation. Moreover, they reported that the disruption of osteoblast-FN interactions suppressed expression of ALP and OC mRNA but had no significant effect on Col I and OPN mRNA expression. In addition, Jimbo et al. studied the effects of plasma FN (pFN) on the osseointegration process in the mice femur. They showed the increased OC mRNA expression at bone-implant interface and found the faster rate of bone formation on the pFN-coated implant surface compared to the control. It is possible that the mechanism of PSS-co-MA on the induction of osteoblast differentiation is due to the rapidly adsorbed proteins on the films. The ability of PSS-co-MA on protein absorption needs further investigation.

Despite the surface property on protein adsorption, the increased differentiation and mineralization observed in this study may result from the direct interaction of cells to the PEM film. PSS-co-MA contains certain amount of carboxyl group from maleic acid which may interact with osteoblast cell surface. Formation of ionic bonds between the carboxyl group of maleic acid and the calcium of hydroxypatite and enamel has been reported. The direct interaction between maleic acid and osteoblast participates in the induction ability of PSS-co-MA film, remain to be elucidated.

**CONCLUSION**

In present study, the formation of PSS-co-MA PEM films had been proposed to improve the titanium surface. Cell adhesion, proliferation, differentiation and in vitro calcium deposition of MC3T3-E1 cultured on PSS-co-MA...
films were investigated. The results showed that PEM films terminating with PSS-co-MA promoted osteoblast differentiation as shown by increasing in ALP activity, expression of OC mRNA/protein and faster rate of calcification. The results suggested the ability of PSS-co-MA-coated PEM surfaces in the enhancement of osteoblast differentiation.

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