

Adlay Polyelectrolyte Multilayer Films Coated on Titanium: Surface Characteristics and MC3T3–E1 Cell Morphology and Proliferation

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Abstract:

Objective: Adlay has been reported to prevent osteoporosis, and promote osteoblast cell proliferation and *in vitro* calcification. However, it has never been used on modified titanium (Ti) surfaces. Hence, the aim of this study was to ameliorate Ti surfaces, by coating with adlay seed extract via the polyelectrolyte multilayer (PEM) film technique.

Material and Methods: Adlay seed extract solution containing 150, 300, 600, or 1500 µg/ml concentrations was coated on Ti discs using a layer-by-layer technique to fabricate PEM films (Ti_Adlay surface). The surface characterizations; including atomic force microscope analysis, contact angle analysis and energy dispersive X-ray analysis were evaluated. The osteoblast cell proliferation on its modified surface was also examined.

Results: Adlay seed extract could increase surface irregularity, roughness, hydrophilicity and carbon composition of Ti surface in all Ti_Adlay groups. At 24, 48 and 72 hours of incubation, the osteoblast cells morphology was similar in all groups. At 24 hours, the viable cell numbers on all Ti_Adlay groups were statistically lower than the uncoated Ti group, while no significant difference was found after 48 and 72 hours of incubation.

Conclusion: Adlay PEM coating on Ti surface could improve the surface properties of Ti in terms of surface roughness, hydrophilicity and surface chemistry. Even though Ti-Adlay surfaces showed no toxic effect on MC3T3–E1, it was unlikely

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to promote osteoblast cell adhesion and proliferation when compared to bare Ti surfaces. Further studies are needed to improve the biological response of Ti_Adlay surfaces to benefit clinical application.

Keywords: adlay, osteoblast cells, polyelectrolyte multilayer films, surface characteristics, titanium

Introduction

Endosseous dental implants are used for replacing tooth loss in edentulous or partially edentulous patients. With its biocompatibility, resistance to corrosion, and high mechanical properties, titanium (Ti) has become the primary biomaterial for dental implants¹. To achieve rapid osseointegration, the current implantology research has attempted to modify the surface properties of Ti implants; in regard to surface energy, surface charge, surface composition, surface topography and surface roughness¹. Several methods have been used to modify the Ti surface; for example, acid or alkaline etching, blasting², plasma spraying³, electrochemical treatment⁴ and bioactive molecules coating^{5,6}. These methods can be used separately or in combination^{7,8}, and such modifications can improve osteoblast functions and mineralization; both *in vitro* and *in vivo*²⁻⁸.

The polyelectrolyte multilayer (PEM) films technique, or the layer-by-layer (LbL) method, is a surface modification technique first introduced by Decher et al. (1992). The PEM films are fabricated by alternating anionic and cationic polyelectrolytes⁹. The electrostatic between opposite ions is considered the major force responsible for the PEM films' stability^{9,10}. PEM films coated on material surfaces could be constructed using synthetic polyelectrolytes, bioactive molecules, DNA, or biopolymer inorganic molecules and particles^{10,11}. A PEM film coating on material surfaces could improve surface topography, surface hydrophilic properties, mechanical properties as well as chemical properties; such as pH, ionic strength and chemical cross-linking¹¹. For Ti implant applications, several biological molecules

including various kinds of polyelectrolytes have been used to construct PEM films coated on Ti surfaces; such as hyaluronic acid, chitosan, arginyl-glycyl-aspartic acid (RGD) peptide, poly(diallyldimethylammonium chloride) (PDADMAC), poly(sodium 4-styrene sulfonate) (PSS), poly(4-styrenesulfonic acid-co-maleic acid) sodium salt (PSS-co-MA), poly(L-lysine) and bone morphogenetic protein 2 (BMP-2). It has been reported that the fabrication of PEM films on Ti surface using these combined molecules or polyelectrolytes; such as (hyaluronic acid/chitosan)₅/RGD peptide⁵, (PDADMAC/PSS)₄/PDADMAC/PSS-co-MA⁶ and [poly(L-lysine)/hyaluronic acid]₂₄/BMP-2¹², improves osteoblast cell adhesion, proliferation, differentiation, maturation as well as mineralization.

Adlay (*Coix lacryma-jobi* L.; coix, Job's tears or Chinese pearl barley) is a tropical plant found in both Eastern and Southeast Asian countries; such as Thailand, China and India¹³. In Chinese medicine, adlay seeds have been used to remedy several symptoms. It can remove heat from the diuretic effect, promote lung and spleen function and can be used in arthritis and diarrhea treatment¹³. Adlay seeds comprise of carbohydrates, proteins, lipids, minerals (phosphorus, magnesium, and potassium), vitamins, carotenoids, phytosterols, polyphenols (phenolic acid and flavonoids) and lactams¹³. Studies have also reported good biological effects from adlay extract; including anti-inflammation¹⁴, antioxidant¹⁵, anti-allergy¹⁶, gastroprotection¹⁷ and anti-cancer effects¹⁸. The effects of adlay on osteoporosis prevention have been demonstrated, and shown that it could reverse the decreased alkaline phosphatase (ALP) activity and calcium level in bones.

Additionally, it was able to decrease tartrate-resistant acidic phosphatase activity in an ovariectomized rat femoral metaphyseal tissue culture model¹⁹ and in an ovariectomized mouse model²⁰. Our previous study demonstrated that cell culture media supplemented with adlay seed extract could promote primary human osteoblast cell proliferation and enhance *in vitro* calcification²¹. However, the biological effects of adlay extract as a biomaterial coating substance have not been reported.

Hence, the objective of this study was to modify the Ti surface by coating it with adlay seed extract, using the PEM technique, to improve the surface property of the Ti surface. The characteristics of adlay PEM film coated on Ti surfaces were investigated; in regard to: surface topography, roughness, surface hydrophilicity and elemental analysis. Furthermore, the morphology and proliferation of osteoblast cells cultured on this modified surface were also examined.

Material and Methods

Titanium disc preparation

A grade II commercially pure Ti bar, with a diameter of 15 mm (KVM Heating Element Co., Ltd., Pathum Thani, Thailand) was used. Specimens were prepared by cutting the Ti bar into Ti discs, at a thickness of 3 mm. A total of 234 Ti discs were prepared, and the surfaces of all specimens were polished using 600–1000 grit silicon carbide papers in a polishing machine (Buehler Ecomet 3, Buehler Ltd., Illinois, USA). This was followed by cleaning with acetone, ethanol and deionized (DI) water, in that order, for 10 minutes in each solution and air drying.

Adlay coated on Ti by PEM film fabrication

A highly cationic polyelectrolyte charged PDADMAC (Aldrich, Missouri, USA), a highly anionic polyelectrolyte charged PSS (Aldrich) and adlay seed extract (Coix lacryma extract powder, Special Natural Products Co., Ltd.,

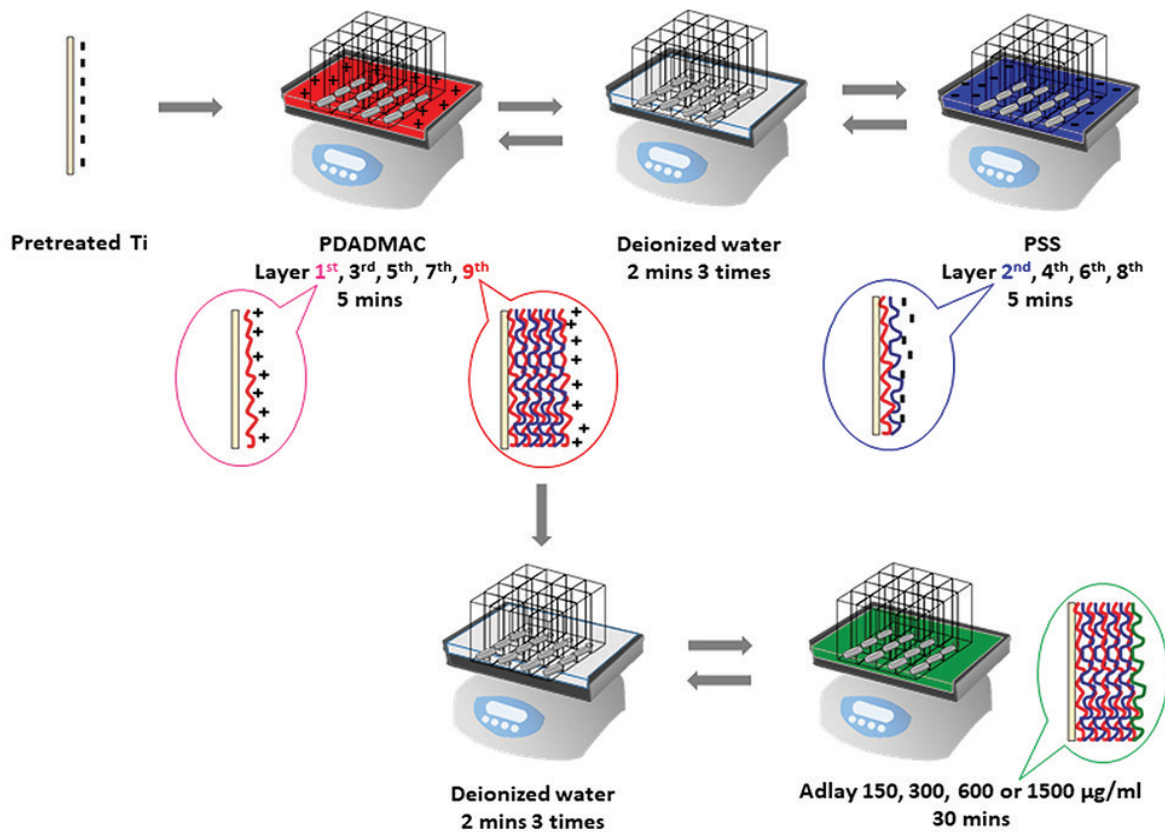
Chonburi, Thailand) were used to fabricate PEM films onto the Ti discs. PDADMAC and PSS were dissolved in DI water and prepared at a concentration of 0.1 mM in 0.1 M NaCl. Adlay seed extract was dissolved in DI water at 150, 300, 600 or 1,500 µg/ml concentrations. The PEM films were fabricated from 9 alternating layers of PDADMAC and PSS, with a final layer of adlay $\{(PDADMAC/PSS)_4/PDADMAC/Adlay\}$ onto Ti discs. Briefly, the pretreated Ti discs were dipped in PDADMAC solution for 5 minutes, triple rinsed with DI water, dipped in PSS solution for 5 minutes and then triple rinsed with DI water. The dipping process was repeated until the ninth layer was formed $[(PDADMAC/PSS)_4/PDADMAC]^6$. For the final layer, the Ti discs were dipped in adlay seed extract solutions; containing 150, 300, 600 or 1500 µg/ml concentrations for 30 minutes, triple rinsed with DI water, and then air dried at room temperature (Figure 1 and Table 1).

Atomic force microscope (AFM) analysis

The AFM (PARK/XE-120, Park Systems Inc, Suwon, Korea) was used to analyze surface topography and surface roughness, using True Non-Contact Mode by a randomized scanning surface area ($1 \times 1 \mu\text{m}^2$) of the Ti discs. The average surface roughness (R_a) and the root mean square of surface roughness (R_q) were reported. The analysis was performed on three specimens of each group.

Contact angle analysis

The surface hydrophilicity of the specimens was determined by measuring the water contact angle using the sessile drops method. The First Ten Angstroms FTA1000B Automated Drop Shape Analyzer software package (First Ten Angstroms, Inc., Virginia, USA) evaluated water contact angles after dropping a 10 µL droplet of DI water onto the Ti surfaces. Three specimens per group were selected for water contact angle measurement.



PDADMAC=poly(diallyldimethylammonium chloride), PSS=poly(sodium 4-styrene sulfonate), PEM=polyelectrolyte multilayer

Figure 1 The fabrication of adlay PEM film coating on Ti discs

Table 1 Control and experimental groups in this study

Group no.	Control and experimental groups	Abbreviations
1.	Uncoated titanium discs	Ti
2.	[(PDADMAC/PSS) ₄ /PDADMAC] coated on Ti discs	Ti_PDADMAC
3.	[(PDADMAC/PSS) ₄ /PDADMAC/adlay 150 µg/ml] coated on Ti discs	Ti_Adlay 150
4.	[(PDADMAC/PSS) ₄ /PDADMAC/adlay 300 µg/ml] coated on Ti discs	Ti_Adlay 300
5.	[(PDADMAC/PSS) ₄ /PDADMAC/adlay 600 µg/ml] coated on Ti discs	Ti_Adlay 600
6.	[(PDADMAC/PSS) ₄ /PDADMAC/adlay 1500 µg/ml] coated on Ti discs	Ti_Adlay 1500

PDADMAC=poly(diallyldimethylammonium chloride), PSS=poly(sodium 4-styrene sulfonate)

Energy dispersive X-ray (EDX) analysis

The chemical composition of each surface specimen was investigated by an EDX system connected to a

scanning electron microscope (SEM; S3000N, Hitachi, Tokyo, Japan). Specimens (n=3) were mounted on a copper plate with carbon tape. Three areas of each specimen were

randomly selected for evaluating surface composition, with all elements analyzed using the EDX processing option. The elements were reported by weight % and atomic %.

Osteoblast cell culture

A mouse calvarial-derived osteoblast-like cell line, MC3T3-E1 (ATCC CRL-2593), was cultured in minimum essential medium (MEM; HyClone MEM/EBSS, HyClone, Utah, USA), which was supplemented with 10% fetal bovine serum (FBS; HyClone), 1% L-glutamine (GlutaMAX™ Supplement, Thermo Fischer Scientific, Massachusetts, USA) and 1% Antibiotic-Antimycotic (Thermo Fischer Scientific), in a 5% CO₂ humidified air chamber; at 37 °C. The medium was changed every other day. The cells' passage at 24 to 30 was used in this study.

MTT assay for cell adhesion and proliferation

The viability of osteoblast cells cultured on all Ti specimen groups was determined by MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), USB Corporation, Ohio, USA) assay, and performed in three-independent experiments in a triplicate set. The Ti discs were sterilized using UV light from the class II Biosafety cabinet (The Esco Airstream, Esco Micro Pte. Ltd., Pennsylvania, USA) for 30 minutes on each side. Specimens were then placed into 12-well tissue culture plates (Corning, New York, USA). MC3T3-E1 cells were cultured on the specimen surfaces at a density of 3×10^4 cells per specimen for 24, 48 and 72 hours. At the end of each incubation period, MC3T3-E1 cells were incubated with 5 mg/ml MTT solution in Dulbecco's Modified Eagle's Medium (DMEM), without phenol red (Gibco, Grand Island, New York, USA) for 30 minutes. The formazan product was dissolved using dimethyl sulfoxide (Merck, Darmstadt, Germany) and measured by the Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific Inc.); at an absorbance wavelength of 570 nm. The percentage of cell viability was then calculated.

SEM analysis for osteoblast cell morphology

MC3T3-E1 cells were seeded onto each Ti disc group, as described above. After reaching the incubating periods, specimens were rinsed twice with phosphate buffered saline, fixed with 3% glutaraldehyde solution (Sigma-Aldrich, Missouri, USA) for 30 minutes, rinsed with 0.1M PBS, dehydrated with a series of 30, 50, 70, 90, and 100% ethyl alcohol and critically dehydrated with 100% hexamethyldisilane (Sigma-Aldrich) for 5 minutes. Each Ti disc was coated with a gold sputter, and the attached osteoblast cell's morphology was examined using SEM (S3000N, Hitachi).

Statistical analysis

Data were reported as the mean \pm standard deviation (S.D.) of three separate experiments. Data were analyzed with the Shapiro-Wilk test to confirm normal distribution, followed by one-way analysis of variance (one-way ANOVA) and multiple comparison analysis (Bonferroni test) using statistical software (IBM® SPSS® Statistic 20 for Windows, IBM corporation, USA). A *p*-value less than 0.05 was considered as a statistically significant difference.

Results

Surface topography and roughness

The surface nanotopography of the specimens is shown in Figure 2. The uncoated Ti group exhibited a smooth appearance, while others showed some extent of irregularity. The increase in nanoscale rugged presented was related to the increase in adlay concentration, which corresponded with the surface roughness data; as shown in Table 2. The statistically significant difference of both R_a and R_q was observed when the Ti and Ti_PDADMAC groups were compared to the Ti_Adlay 300, Ti_Adlay 600, and Ti_Adlay 1500 groups (*p*-value<0.05). However, the R_a and R_q of the Ti group had no significant difference when compared to the Ti_PDADMAC and Ti_Adlay 150 groups (*p*-value>0.05).

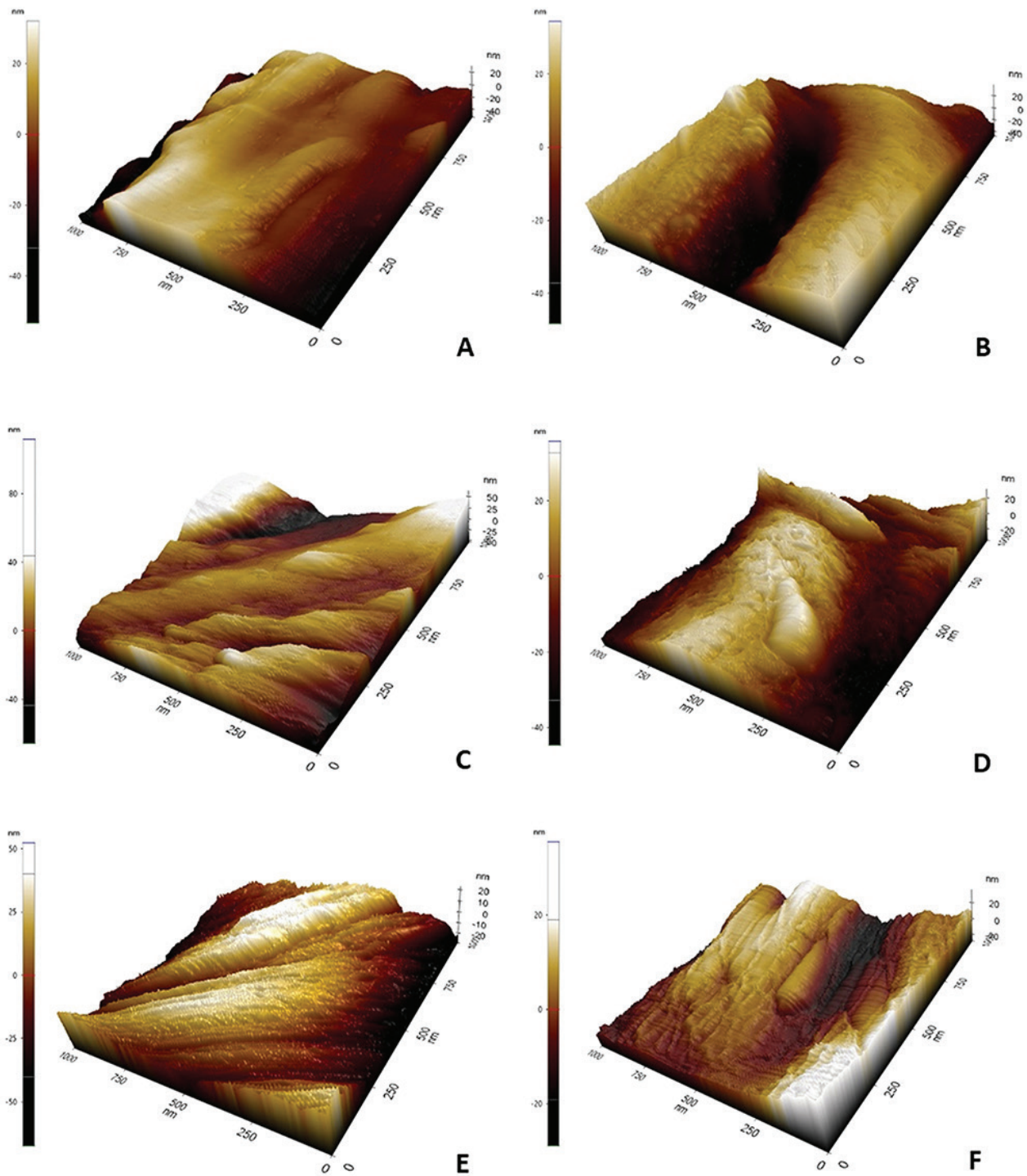


Figure 2 The surface topography of Ti (A), Ti_PDADMAC (B), Ti_Adlay 150 (C), Ti_Adlay 300 (D), Ti_Adlay 600 (E) and Ti_Adlay 1500 (F) groups.

Table 2 R_a and R_q values of all groups







Group of specimens	R_a (nm)	R_q (nm)
Ti	2.45±0.84	3.13±1.03
Ti_PDADMAC	4.01±0.41	5.27±0.54
Ti_Adlay 150	5.55±0.94	7.32±0.99
Ti_Adlay 300	8.87±0.69 ^{a,b}	10.90±1.80 ^{a,b}
Ti_Adlay 600	9.53±2.26 ^{a,b}	11.83±2.41 ^{a,b}
Ti_Adlay 1500	9.36±2.10 ^{a,b}	11.05±2.53 ^{a,b}

Data are presented as mean±S.D.
a, b, indicated a significant difference compared to Ti and Ti_PDADMAC, respectively (p-value<0.05).

Surface hydrophilicity

The water contact angle of all specimen groups is shown in Table 3. No significant difference was found between the Ti and Ti_PDADMAC groups (p-value>0.05), while a significant decrease in water contact angle was found when comparing the Ti group to all other Ti_Adlay groups (p-value<0.05).

Table 3 The water contact angle values

Group of specimens	Water contact angle (degree)
Ti	 79.33±7.57
Ti_PDADMAC	 66.92±2.66
Ti_Adlay 150	 50.38±7.55 ^a
Ti_Adlay 300	 52.85±2.75 ^a
Ti_Adlay 600	 54.20±5.10 ^a
Ti_Adlay 1500	 49.70±7.83 ^a

Data are presented as mean±S.D.
a indicated a significant difference between Ti and the other groups (p-value<0.05).

Elemental analysis

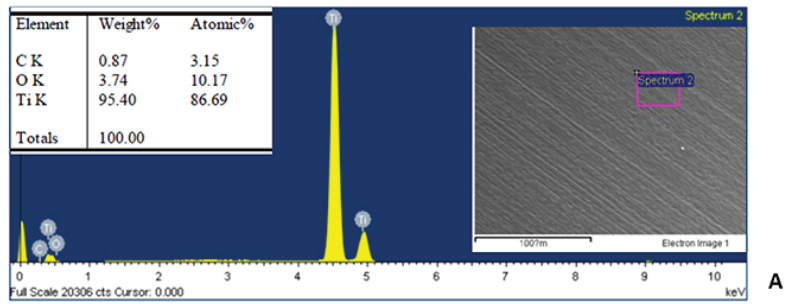
The weight and atomic percentages of the chemical compositions on the specimen surfaces were examined by EDX (Figure 3). EDX results demonstrated that the main elements presented on all specimens were C (carbon), O (oxygen) and Ti (Figure 3A). The percentage of the C element was significantly increased in all groups when compared to the uncoated Ti surfaces (p-value<0.05) (Figure 3B). In contrast, no significant difference in Ti and O elements was found among all groups (p-value>0.05) (Figure 3C, 3D).

Cells viability, adhesion, and proliferation

MTT assay showed that osteoblasts could adhere and proliferate on all specimen groups in a time-dependent manner (Figure 4A). At 24 hours, the highest number of viable cells was found in the Ti group; with statistically significant (p-value<0.05) (Figure 4B). At 48 hours, a significant difference in the number of viable cells was found when comparing Ti, Ti_Adlay 300 and Ti_Adlay 600 to the Ti_PDADMAC group (p-value<0.05) (Figure 4C). At the end of the 72-hour incubation period, no significant difference in the number of viable cells was found among all groups (p-value>0.05) (Figure 4D).

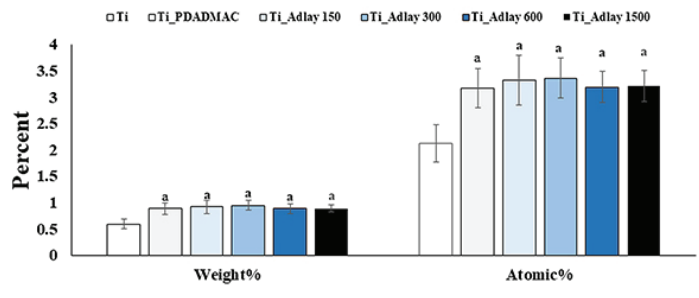
Cell morphology

The SEM images demonstrated a similar change in MC3T3-E1 cell morphology over time on all surfaces (Figure 5). At 24 hours, MC3T3-E1 cells were well adhered to and had spread. At 48 hours, cells were elongated, flattened and beginning to form contact with adjacent cells. After 72 hours of incubation, most of the MC3T3-E1 cells were fully spread. Moreover, the number of cells adhered on all surfaces was increased over time: corresponding to the MTT results.



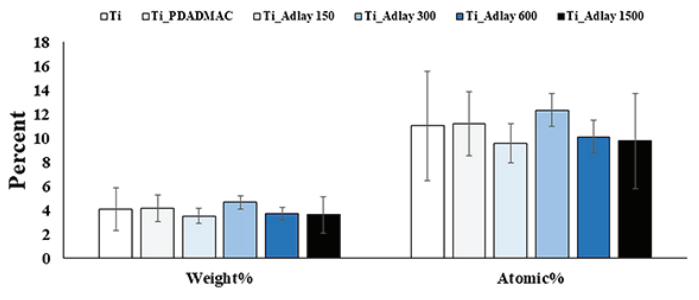
A

Carbon



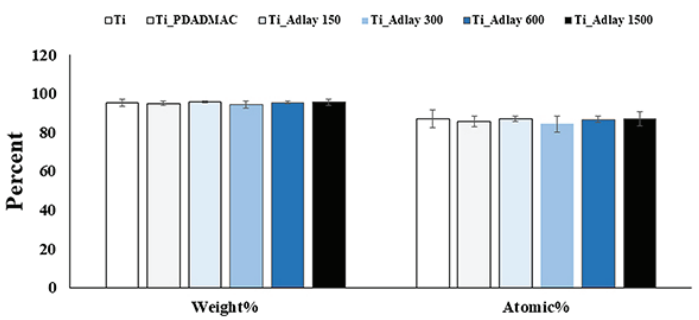
B

Oxygen



C

Titanium



D

Figure 3 The chemical compositions on the specimens surface and SEM images with the rectangle selected EDX field (A). The elemental analysis of carbon (B), oxygen (C) and Titanium (D). Data are presented as mean±S.D. (n=3). a indicated significant difference compared to the Ti group (p-value<0.05).

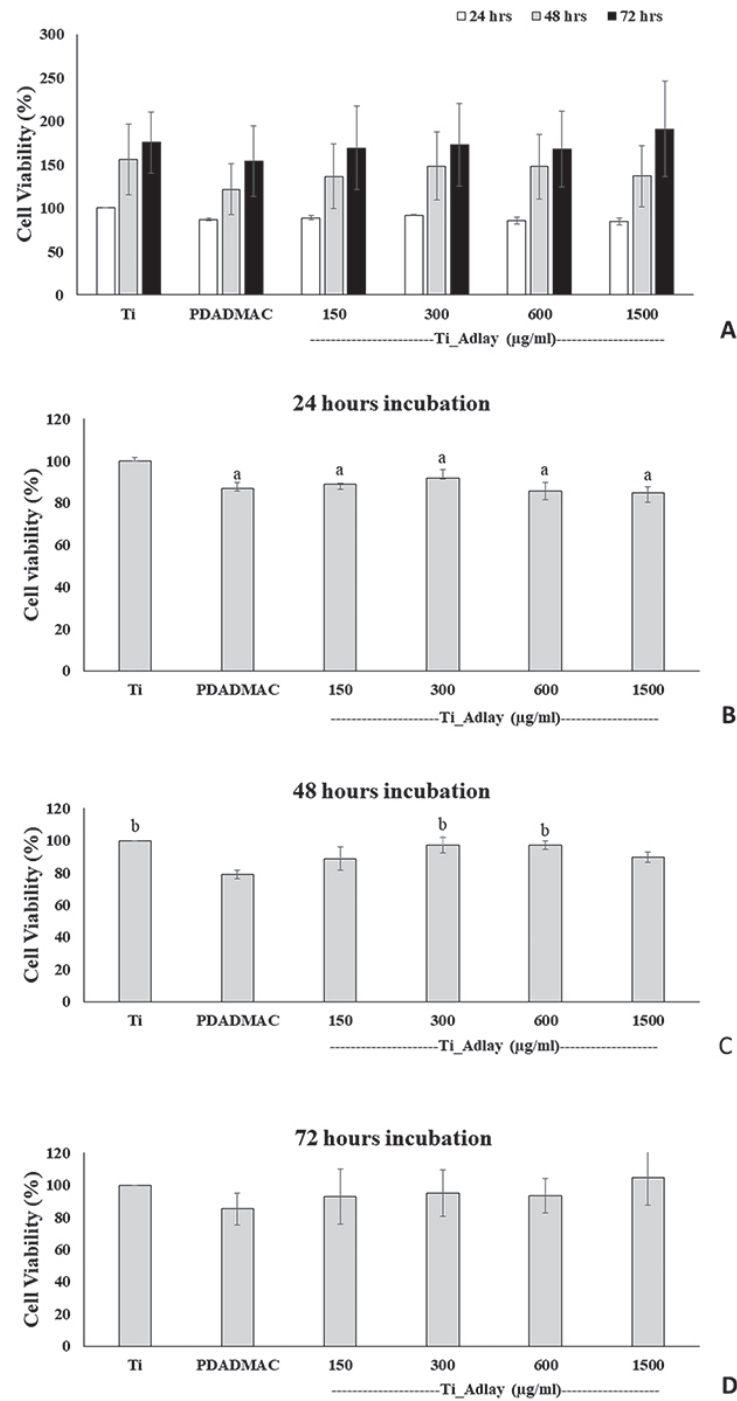


Figure 4 The viability of MC3T3-E1 cells cultured on the Ti, Ti_PDADMAC, Ti_Adlay 150, Ti_Adlay 300, Ti_Adlay 600 and Ti_Adlay 1500 groups (A) at 24 (B), 48 (C) and 72 (D) hours of the incubation periods. Data are presented as mean±S.D. (n=3). a, b indicated significant difference compared to the Ti and Ti_PDADMAC groups, respectively (p-value<0.05)

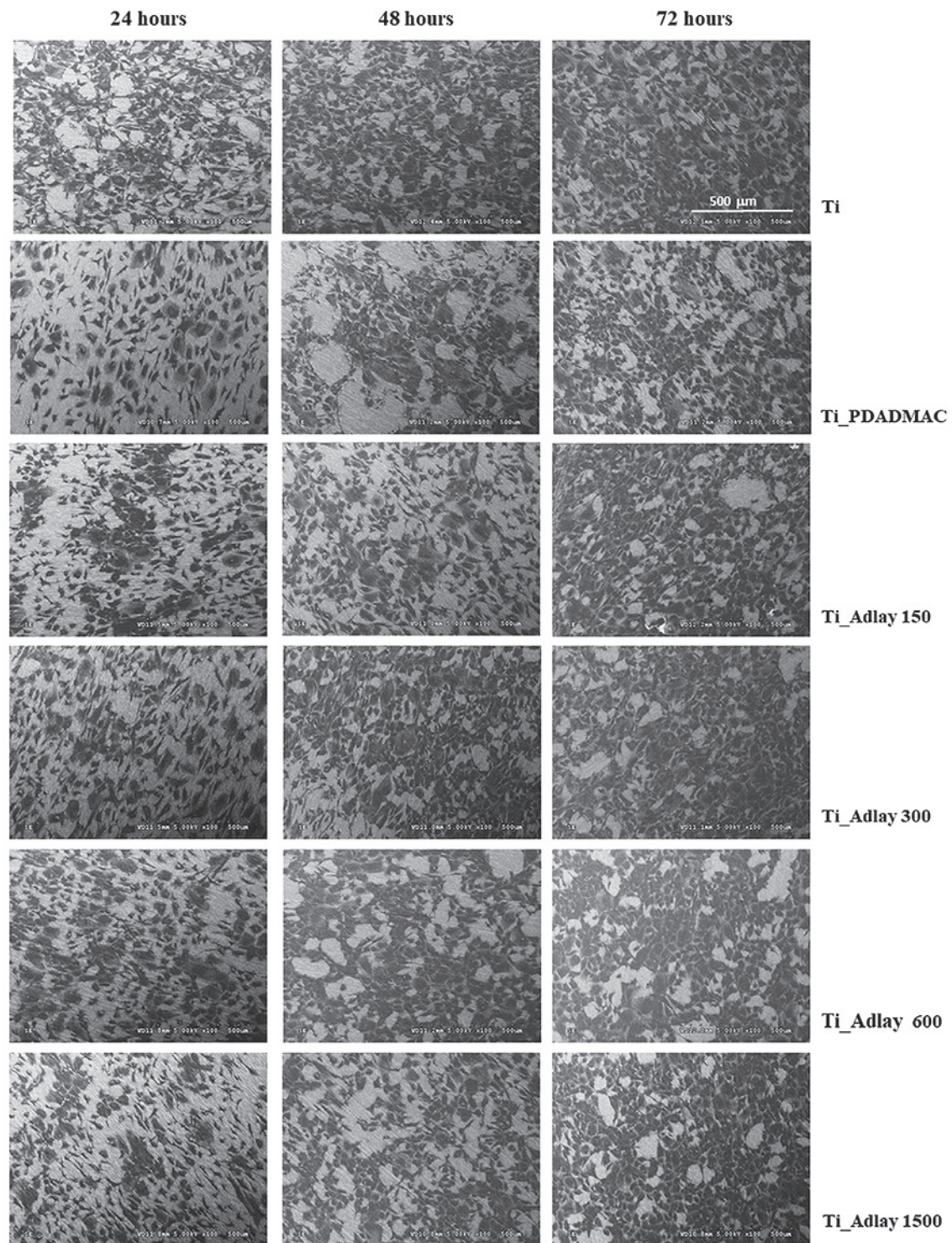


Figure 5 Scanning electron micrograph of osteoblast cells at 100X magnification from all groups (Ti, Ti_PDADMAC, Ti_Adlay 150, Ti_Adlay 300, Ti_Adlay 600, and Ti_Adlay 1500) at 24, 48 and 72 hours of the incubation periods.

Discussion

In bone research, it has been reported that adlay has a preventive effect on osteoporosis. Nonetheless, few studies have stated adlay's effects on promoting osteogenesis. Our previous studies demonstrated the positive impact of adlay extracts on primary human osteoblasts in promoting cell proliferation and *in vitro* calcification²¹. Moreover, there is a lack of research on using adlay as a surface modification material; especially for Ti implant surface modification, to enhance osseointegration. In this study, the Ti surface was modified by coating it with adlay seed extracted using the PEM film technique. This coating method was selected because it is a simple, inexpensive, fast, stable and efficient technique to construct biologically active surfaces¹¹. Various polyelectrolyte solutions as well as biological molecules can be used to fabricate PEM films; such as poly(L-lysine) and hyaluronic acid^{5,12}. This study used PDADMAC and PSS to fabricate PEM films, with the final layer being the adlay extract. As PDADMAC and PSS are strong polyelectrolytes, their ionic charges are largely independent of pH conditions²². Additionally, the hydrophilic property of the polycations of PDADMAC will also assist in the addition of the next PSS layer²². These result in the formation of PEM films that can be stably controlled for thickness and surface roughness²². Furthermore, our previous studies reported the successful fabrication of 9 alternating layers of PDADMAC and PSS films, with other polyelectrolytes deposited on the final layer^{6,22-24}. This present study successfully fabricated PEM films with a final layer of adlay seed extract coating on Ti discs to improve Ti surface properties; including topography, surface roughness, hydrophilicity and surface chemistry. As the biological responses to PEM films depended on the outer layer of PEM films^{5,25}, the outermost adlay layer could influence the osteoblast response and osteogenesis.

The degree of osseointegration of implant materials is largely influenced by surface properties; such as

roughness, hydrophilicity, charge and surface chemistry^{1,26,27}. Surface roughness is one of the features determining the associations between surface properties and osteoblast cell behaviors. In this study, the surface roughness of all Ti_Adlay groups was higher than the uncoated Ti group in a nanometer-scale (range 2–7 nm); wherein, the increase in surface roughness correlated with the increase in adlay concentration. This could have resulted from increased adlay particles aggregating on the PEM films. However, when considering the relationship between surface roughness and cell viability, the increase in surface roughness is unlikely to promote osteoblast cell adhesion or proliferation after 24–72 hours of incubation. The probable explanation is that the difference in surface roughness between 2–17 nm has relatively little effect on the osteoblast cell adhesion and proliferation. Although, several studies have reported the effects of nanoscale surface roughness on osteoblast cell functions, surface roughness values differed from this present study. Webster et al.²⁸ reported that the range of R_a between 17–32 nm could affect osteoblast cell adhesion. Additionally, Vandrovцова et al.²⁹ showed that the R_a value in a range of 40–200 nm could not affect the number of viable MG63 cells on the first day of incubation; however, the cell population density was decreased with increasing surface roughness after 7 days of incubation. In contrast, Giner et al.'s study⁸ did not find a difference in the number of cells cultured on Ti, with an average roughness (S_a , arithmetical mean height) value of 1.36 μm when compared to those of 0.18 μm at 3-days incubation; however, there was a significant increase of cell numbers observed at day 7. Osathanon et al.³⁰ reported an association between surface roughness and MC3T3-E1 cell attachment and spreading. As mentioned, the surface roughness of each study was different, and therefore the osteoblast's responses were also different. Until now, the ideal surface roughness value, which could optimally promote cell adhesion and proliferation, is still unspecified. In the Ti surface modification process,

surface roughness is not the only property that has been altered. As such, it is impossible to consider each factor's effect separately.

The surface hydrophilicity of materials is also an important factor in determining cellular responses and behaviors. This current study revealed that adlay PEM films coated on Ti discs could significantly enhance surface hydrophilicity compared to the uncoated Ti surfaces. The increase in surface hydrophilicity might be from the functional groups of adlay elements; such as the -OH group of carbohydrates and polyphenols (vanillic acid) and -NH₂ or -COOH groups of amino acid¹³. Although, the relationship between surface hydrophilicity and osteoblast responses is still controversial, several studies have stated that increased hydrophilicity of the Ti surface could promote cell interactions regarding adhesion, proliferation and differentiation^{22,31-34}. Altankov et al.³¹ suggested that the increased surface hydrophilicity could accordingly increase cell proliferation. Furthermore, Van Wachem et al.³⁴ demonstrated that the hydrophilicity surface with a water contact angle of 13°-44° would have the most conductive effect on cell proliferation. On the contrary, studies by Nijhuis et al.³⁵ and Osathanon et al.³⁰ reported the absence of an association between surface hydrophilicity to osteoblast cell behaviors. Similarly, this study also found that although adlay PEM films could increase Ti surface hydrophilicity, they did not seem to affect osteoblast cell adhesion and proliferation.

The surface chemistry of materials could also affect osteoblast functions and activities. Several bioactive components of adlay; especially polyphenols (phenolic acid, flavonoids) and vitamins, may positively affect osteogenesis in various ways¹³. According to Xiao et al.'s study³⁶, vanillic acid extraction from elderberries had osteogenesis effects on osteoblast-like cells MCT3T-E1 and UMR 106. Even though it did not promote the proliferation of MC3T3-E1, vanillic acid could significantly enhance the ALP activity of both

MC3T3-E1 and UMR 106 cells. It also increased the mRNA expression of runt-related transcription factor 2 (Runx2), ALP and osteocalcin in UMR 106 cells. Quercetin and rutin, the flavonoids found in adlay, are also reported to positively affect osteogenesis. Srivastava et al.'s study³⁷ stated that quercetin and rutin could increase proliferation, stimulate ALP activity, and *in vitro* calcification as well as enhance osteopontin, osterix and Runx-2 mRNA expressions of mouse bone marrow mesenchymal stem cells. These studies suggested the positive roles of vanillic acid, quercetin, and rutin in osteoblast activities^{36,37}. However, only a small amount of phenolic acid and flavonoids are found in adlay compositions¹³; wherein, the major components of adlay are carbohydrates (approximately 70%). Therefore, the effect of these bioactive substances could be shielded. In the adlay PEM film fabrication procedure, carbohydrates were probably dissolved into the water and coated onto the Ti surfaces. This was confirmed by the increase of carbon on the Ti surfaces in all Ti_Adlay groups when analyzed with EDX. The carbohydrate coating might reduce the Ti surface stiffness; thus, resulting in osteoblast cell adhesion reduction³⁸. Furthermore, the carbohydrate formation might entrap other compositions of adlay into itself, preventing the bioactive components from forming direct contact with the Ti surface and becoming inactive molecules. This might explain why adlay-PEM films could not promote osteoblast adhesion and proliferation in this study. On the other hand, our previous study found that culture media containing adlay seed extracts could promote primary human osteoblast cell proliferation and *in vitro* calcification²¹. A possible explanation is that these bioactive molecules might be dissolved in the culture media, becoming active forms that could directly contact cultured cells and promote osteoblast functions. In addition, the concentration of adlay in PEM films and culture media was calculated differently due to different experimental settings. Moreover, many studies found that even if the surface modification of biomaterials did not

promote cell adhesion and proliferation, it could encourage osteoblast cell differentiation and mineralization faster^{8,22}. As a result, further investigations are needed to clarify these issues.

Conclusion

Adlay PEM film coating on the Ti surface could alter the surface properties of Ti in terms of surface roughness, hydrophilicity and surface chemistry. Although these properties were altered in a way that seemed to positively support osteoblast activity, no significant difference in osteoblast cell morphology, adhesion nor proliferation was observed when compared to an uncoated Ti surface. Further studies; including adlay concentration adjustment, the differentiation and mineralization of osteoblast cells cultured on adlay PEM films coated on Ti discs, or alteration of the biological effects of adlay PEM films by removing carbohydrates from PEM films, are interesting aspects for ongoing research.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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