ผลของพอลิโฟร์สไตรีนซัลโฟนิคแอซิดโคมาลิอิคแอซิดโซเดียมซอล์ทพอลิอิเล็กโตรไลท์มัลติเลเยอร์ฟิล์ม ต่อการสร้างกระดูกบนไทเทเนียม: การศึกษาในห้องปฏิบัติการและในสัตว์ทดลอง

นางสาววัชวดี หุ่นวิจิตร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

THE EFFECT OF POLY(4-STYRENESULFONIC ACID-CO-MALEIC ACID) SODIUM SALT POLYELECTROLYTE MULTILAYER FILMS TO BONE FORMATION ON TITANIUM IN VITRO AND IN VIVO STUDY

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วัชวดี หุ่นวิจิตร : ผลของพอลิโฟร์สไตรีนซัลโฟนิคแอซิดโคมาลิอิคแอซิดโซเดียมซอล์ทพอลิอิเล็กโตรไลท์มัลติเลเยอร์ฟิล์มต่อการสร้างกระดูกบนไทเทเนียม:การศึกษาในห้องปฏิบัติการและในสัตว์ทดลอง
(THE EFFECT OF POLY(4-STYRENESULFONIC ACID-CO-MALEIC ACID) SODIUM SALT POLYELECTROLYTE MULTILAYER FILMS TO BONE FORMATION ON TITANIUM IN VITRO AND IN VIVO STUDY)
อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ทพ.ดร. แมนสรวง อักษรนุกิจ,
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การศึกษานี้ศึกษาถึงการตอบสนองของเซลล์สร้างกระดูกต่อไทเทเนียมที่ปรับปรุงพื้นผิวด้วยฟิล์มบางหลายชั้นของพอลิอิเล็กโทรไลท์ หรือพีอีเอ็มฟิล์ม ซึ่งใช้พอลิไดอัลลิวไดแมททิวแอมโมเนียมคลอไรด์ (พีดีเอดีเอ็มเอซี), พอลิโซเดียม-4-สไตรีนซัลโฟเนท (พีเอสเอส) และพอลิ-4-สไตรีนซัลโฟนิคแอซิดโคมาเลอิคแอซิดโซเดียมซอลท์ (พีเอสเอสโคเอ็มเอ) โดยเป็นการศึกษาทั้งในห้องปฏิบัติการและในสัตว์ทดลอง การศึกษาในห้องปฏิบัติการ ประกอบด้วยการศึกษาลักษณะทางกายภาพของชิ้นไทเทเนียมที่เคลือบด้วยพีอีเอ็มฟิล์ม ด้วยกล้องจุลทรรศน์อิเล็กตรอนชนิดส่องกราด กล้องจุลทรรศน์แรงอะตอม และมุมสัมผัสที่ผิว(ระหว่างน้ำและไทเทเนียม) ตามลำดับ รวมทั้งศึกษาการสร้างไฟบรินหลังหยดเลือดลงบนไทเทเนียม ศึกษาการแสดงออกของยีนของเซลล์สร้างกระดูกเอ็มซีสามทีสาม-อีหนึ่ง โดยการวิเคราะห์ปริมาณเอ็มอาร์เอ็นเอด้วยวิธีอาร์ที-พีซีอาร์ และการตกตะกอนของแคลเซียมด้วยการย้อมสีอลิซารินเรด-เอส ส่วนการศึกษาในสัตว์ทดลอง จะทำโดยการฝังลวดไทเทเนียมในกระดูกต้นขาของหนูวิสตาร์ แล้ววิเคราะห์ปริมาณกระดูกที่สร้างขึ้นใหม่ จากผลการทดลองพบว่า ไทเทเนียมที่เคลือบด้วยพีเอสเอสโคเอ็มเอมีความชอบน้ำเพิ่มสูงขึ้น แต่ไม่มีความแตกต่างของความหยาบพื้นผิวเมื่อเทียบกับกลุ่มควบคุม นอกจากนี้ ไทเทเนียมเคลือบ ยังรองรับการสร้างไฟบรินบนพื้นผิวที่เคลือบได้เร็วและมากกว่ากลุ่มควบคุมที่เวลา 5 นาที ในการศึกษาการแสดงออกของยีน พบว่า การแสดงออกของยีนคอลลาเจนชนิดที่หนึ่งเพิ่มขึ้นอย่างมีนัยสำคัญในวันที่5 ในขณะที่ออสตีโอพอนทิน โบนไซอะโลโปรตีน และออสตีโอแคลซินเพิ่มขึ้นในวันที่10 และติดสีย้อมอลิซารินเรด-เอส ที่มากกว่าในวันที่15 บนไทเทเนียมที่เคลือบด้วยพีเอสเอสโคเอ็มเอเมื่อเทียบกับกลุ่มควบคุม ปริมาณกระดูกที่สัมผัสผิวลวดไทเทเนียม (โบนอิมพลานคอนแทค) ที่ถูกเคลือบด้วยพีเอสเอสโคเอ็มเอเพิ่มขึ้นอย่างมีนัยสำคัญเทียบกับกลุ่มควบคุมในหนูเมื่อ 2 สัปดาห์ อย่างไรก็ตามไม่มีความแตกต่างกันของปริมาณกระดูก (โบนโวลุม) ระหว่างกลุ่ม โดยสรุปไทเทเนียมที่ปรับปรุงพื้นผิวด้วยพีเอสเอสโคเอ็มเอพีอีเอ็มฟิล์มเร่งการสร้างกระดูก เทคนิคนี้อาจได้รับเลือกใช้ในการพัฒนาผิวรากเทียมเพื่อเร่งการสร้างกระดูกบนผิวของรากเทียม

ภาควิชา.....ทันตกรรมประดิษฐ์.....ลายมือชื่อนิสิต.....
สาขาวิชา.....ทันตกรรมประดิษฐ์.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก……
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5276127232 : MAJOR PROSTHODONTICS
KEYWORDS : POLY(4-STYRENESULFONIC ACID-CO-MALEIC ACID) SODIUM SALT / POLYELECTROLYTE MULTILAYERS / TITANIUM / MC3T3-E1 / WISTAR RATS

WATCHAWADEE HOONWICHIT: THE EFFECT OF POLY(4-STYRENESULFONIC ACID-CO-MALEIC ACID) SODIUM SALT POLYELECTROLYTE MULTILAYER FILMS TO BONE FORMATION ON TITANIUM IN VITRO AND IN VIVO STUDY. ADVISOR: ASSOC.PROF. MANSUANG ARKSORNNUKIT, D.D.S., M.S.,PhD., CO-ADVISOR: PROF. PRASIT PAVASANT, D.D.S., PhD., 90 pp.

This study was to examine both *in vitro* and *in vivo* responses of osteoblast on titanium (Ti) coated with {(PDADMAC/PSS)$_4$/PDADMAC}PSS-co-MA polyelectrolyte multilayer (PEM) films formed by poly(diallyldimethylammonium chloride) (PDADMAC), poly(sodium 4-styrene sulfonate) (PSS) and poly(4-styrenesulfonic acid-co-maleic acid)sodium salts (PSS-co-MA) to generate PEM films. *In vitro* study included the study of physical characteristics using scanning electron microscope, atomic force microscopy and contact angle measurement, respectively. Fibrin clot formations, utilizing whole blood dropped, on Ti were investigated. Gene expressions of MC3T3E1-osteoblast cells were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and *in vitro* calcifications were detected using alizarin red-S staining. Titanium pins were implanted into the Wistar rat femurs and new bone formations were confirmed by histomorphometric analysis. Results showed PSS-co-MA coated Ti surface had a better hydrophilic property however no change in surface roughness was detected compared to the control. The amount of fibrin formation on coated surface was higher than that on the control. The expressions of type-I collagen were significantly increased at day-5 while the expressions of osteopontin, bone sialoprotein and osteocalcin increased at day-10. Higher alizarin red-S staining was observed at day-15 on coated Ti compared to the control. The bone-to-implant contact around the coated Ti pins significantly increased compared to the control in the rats at 2 weeks. However, no significant differences in bone volume were observed among the different groups. In conclusions, modified Ti surface by PSS-co-MA PEM films accelerates the bone formations. This technique may be the candidate to improve dental implant surface for accelerating osseointegration.

Department : Prosthodontics Student's Signature .....

Field of Study : Prosthodontics Advisor's Signature .....

Academic Year : 2011 Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

AFM	Atomic force spectroscopy
Al	Aluminium
ALP	Alkaline phosphatase
BIC	Bone-to-implant contact
BMP	Bone morphogenetic protein
BSP	Bone sialoprotein
BV	Bone volume
CaP	Calcium phosphate
Chi	Chitosan
Col-I	Type I collagen
Cp Ti	Commercially pure titanium
Gel	Gelatin
LbL	Layer-by-layer
HA	Hydroxyapatite
OC	Osteocalcin
OPN	Osteopontin
PDADMAC	Poly (diallyldimethylammonium chloride)
PAH	poly(allylamine hydrochloride)
PBS	Phosphate buffered saline
PEI	poly(ethyleneimine)
PEM	Polyelectrolyte multilayer
PSS	Poly (sodium 4-styrene sulfonate)
PSS-co-MA	Poly(4-styrenesulfonic acid-co-maleic acid) sodium salt
qRT-PCR	Quantitative reverse-transcription polymerase chain reaction
Ra	Average surface roughness
RGD	Arginine-glycine-aspartic acid
Rq	Root mean square roughness
SEM	Scanning electron microscope

Ti	Titanium
V	Vanadium

CHAPTER I
INTRODUCTION

The dental implant is one of the most efficient therapies for the replacement of missing teeth. The titanium (Ti) and titanium alloys have been used as implant materials due to their biocompatibility, ability to form a direct bone-to-metal interface (osseointegration), high-strength, low weight, and excellent corrosion resistance [1-3]. However, modification of Ti surface in order to achieve a more rapid, stabilization and integration of dental implant depends on many factors such as bone (quality of the host bone, site of bone), dental implant (material, shape, design, surface properties, surface chemistry or surface composition), surgical technique (skill of the surgeon, drilling technique), time and mechanical loading. Therefore, several attempts have been reported in order to modify and improve the properties of the implant surface such as mechanical modification by blasting [4-6] or acid etching [4-6]; chemical modification by plasma-sprayed hydroxyapatite [4, 7] or bioceramic coating [5] or coating with extracellular bone matrix component (collagen [8, 9], RGD peptide [9-11], chitosan [12], etc.), forming oxide layers by electrochemical anodization [13-15] or sol-gel [16] and polyelectrolyte multilayer (PEM) films technique [17-19].

The build-up of PEM films is one of the techniques to modify surface of implant materials in order to enhance their bioactive abilities through the adsorption of protein or other biological molecules capable of transmitting signals to contacting cells [17-20]. The principles of this technique are based on a layer-by-layer deposition method (LbL) with alternatively of positively and negatively charged polyelectrolytes, resulting in multilayer films [21]. The electrostatic attraction of opposite charged between layer to layer and layer to substrate provided a more stable force than physical adsorption. The advantages of this technique are inexpensive and versatile that the different materials and different types of polyelectrolytes can be incorporated in a sample procedure [17, 21].

Recently, Angwarawong *et al.* [22] introduced PEM films generating from Poly(diallyldimethylammonium chroride) (PDADMAC; positively charged), Poly(sodium4-styrene sulfonate) (PSS; negatively charged) and Poly(4-styrenesulfonic acid-co-maleic acid) (PSS-co-MA; negatively charged) to form a [{(PDADMAC/PSS)$_4$/PDADMAC}PSS-co-MA] PEM films on glass surface. Although the in vitro results indicating that this PEM film enhances bone formation, the question remains on the actual effect of PSS-co-MA film when coated on the other materials.

Therefore, the objectives of this study were to investigate the osteoblast response to PSS-co-MA PEM films coated titanium on bone formation both of *in vitro* and *in vivo* studies.

RESEARCH QUESTIONS

1. Whether {(PDADMAC/PSS)$_4$/PDADMAC}PSS-co-MA PEM films on cp titanium affect the surface characteristics of titanium surface.
2. Whether {(PDADMAC/PSS)$_4$/PDADMAC}PSS-co-MA PEM films on cp titanium support the fibrin formation.
3. Whether {(PDADMAC/PSS)$_4$/PDADMAC}PSS-co-MA PEM films on cp titanium support bone formation *in vitro.*
4. Whether {(PDADMAC/PSS)$_4$/PDADMAC}PSS-co-MA PEM films on cp titanium support bone formation *in vivo.*

RESEARCH OBJECTIVES

1. To examine the surface characteristics of cp titanium when coated with {(PDADMAC/PSS)$_4$/PDADMAC}PSS-co-MA PEM films.
2. To examine the fibrin formation on {(PDADMAC/PSS)$_4$/PDADMAC}PSS-co-MA PEM films coated on cp titanium
3. To examine the bone formation of osteoblasts grown on {(PDADMAC/PSS)$_4$/PDADMAC}PSS-co-MA PEM films coated on cp titanium *in vitro*.
4. To evaluate the bone formation of {(PDADMAC/PSS)$_4$/PDADMAC}PSS-co-MA PEM films coated on cp titanium implant *in vivo.*

RESEARCH HYPOTHESIS

1. {(PDADMAC/PSS)$_4$/PDADMAC}PSS-co-MA PEM films coating affect to the degree of roughness and hydrophilic of cp titanium surface.
2. {(PDADMAC/PSS)$_4$/PDADMAC}PSS-co-MA PEM films coating can enhance fibrin formation on cp titanium.
3. {(PDADMAC/PSS)$_4$/PDADMAC}PSS-co-MA PEM films coating can enhance bone formation on cp titanium *in vitro.*
4. {(PDADMAC/PSS)$_4$/PDADMAC}PSS-co-MA PEM films coating can enhance bone formation on cp titanium *in vivo.*

KEY WORDS: *Poly(4-styrenesulfonic acid-co-maleic acid) sodium salt (PSS-co-MA), Polyelectrolyte multilayers (PEM), Titanium, MC3T3-E1, Wistar rats.*

RESEARCH DESIGN

Laboratory and animal experimental research

EXPECTED BENEFITS

The surface modification of titanium by PSS-co-MA PEM films coating may enhance direct bone formation on titanium surface. This knowledge will benefit patient whom have quality and quantity is not suitable for primary implant stability.

CHAPTER II

REVIEW OF RELATED LITERATURES

During the last decades, the replacement of missing teeth with dental implant has become a widely accepted and routinely used treatment modality for the rehabilitation and fully edentulous patients, with success rates often reported at greater than 90% [4, 5, 7, 23].

There are many materials that were used for bony reconstruction. They can be divided in three groups according to their compatibility in bony tissue. These are biotolerant, bioinert and bioactive (show in Table 2.1) [24]. Bioinert materials do not release any harmful substance and therefore do not reveal the adverse tissue reaction. Titanium and titanium alloys are recognized as being bioinert and used in both dental and orthopedic surgery [25].

Table2.1 Grouping of materials for bony reconstruction according to their compatibility in bony tissue[24]

Degree of compatibility	Typical reactions of bony tissue	Materials
Biotolerant	Implants separated from adjacent bone by a soft tissue layer along most of the interface; distant osteogenesis.	Stainless steels, PMMA bone cements and Cobalt-based alloy
Bioinert	Direct contact to bone tissue; contact osteogenesis.	Alumina ceramics, Zirconia ceramics, Titanium, Tantalum, Niobium
Bioactive	Bonding to bony tissue, in the sense of a gluing effect; bonding osteogenesis.	Calcium-phosphate containing glasses and glass ceramics, Hydroxyapatite (HA) and Tri-calcium phosphate ceramics

Implant materials

For medical application titanium and titanium alloys have been used since the 1960s [3, 26, 27]. Titanium and titanium alloys are mostly used as dental implants due to their biocompatibility, ability to form a direct bone-to-metal interface (osseointegration), high-strength, low weight, and excellent corrosion resistance [1, 3, 12].

Many clinicians recognize only two types of titanium implant biomaterials: commercially pure (cp) titanium and titanium alloy. Among these two general groups, however, are six distinct materials defined by the American Society for Testing and Material (ASTM) (show in Table 2.2). ASTM Committee F-4 on Materials for Surgical Implants recognizes four grades of commercially pure titanium and two titanium alloys. The two alloys are Ti-6Al-4V and Ti-6Al-4V extra low interstitial (ELI). All six of these materials are commercially available [1].

Another material was proposed for using as dental implant material. Stainless steel, particularly 316L stainless steel, continues to be used as an implant material for bone plate and screws. This material is stronger, cheaper, and easier to be machined. However, its corrosion property is inferior to titanium [1] and it cannot create direct contact to bone surface [24]. For these reasons, it has not been approved as a dental implant material [1].

Table2.2 Composition of cp Titanium and Alloys (weight percent)[1]

Titanium	N	C	H	Fe	O	Al	V	Ti
cp grade I	0.03	0.10	0.015	0.02	0.18	-	-	balance
cp grade II	0.03	0.10	0.015	0.03	0.25	-	-	balance
cp grade III	0.03	0.10	0.015	0.03	0.35	-	-	balance
cp grade IV	0.03	0.10	0.015	0.05	0.40	-	-	balance
Ti-6Al-4V alloy	0.05	0.08	0.015	0.30	0.20	5.50-6.75	3.50-4.50	balance
Ti-6Al-4V ELI alloy	0.05	0.08	0.012	0.10	0.13	5.50-6.50	3.50-4.50	balance

At temperatures up to 882°C, pure titanium exists as a hexagonal close-packed atomic structure (alpha phase). Above this temperature through the melting point at 1,665°C, the structure is body-centered cubic (beta phase) [1, 26]. In Ti-6Al-4V, vanadium stabilizes the beta phase, so that it exists as a combination of alpha and beta phase. This combination of phase gives the alloy strength. Additional strength gained from dissolved oxygen is inconsequential compared with the effect

of vanadium. Because of this, the ELI alloys are sometimes used. “Extra low interstitial” describes the low levels of oxygen dissolved in interstitial sites in the metal. With lower amounts of oxygen and iron residuals in the ELI alloys, ductility improved slightly [1].

Table2.3 Mechanical properties of selected materials [1]

Material	Modulus (GPa)	Ultimate Tensile Strength (MPa)	Yield Strength (MPa)	Elongation (%)	Density (g/cc)
cp grade I Ti	102	240	170	24	4.5
cp grade II Ti	102	345	275	20	4.5
cp grade III Ti	102	450	380	18	4.5
cp grade IV Ti	104	550	483	15	4.5
Ti-6Al-4V ELI	113	860	795	10	4.4
Ti-6Al-4V	113	930	860	10	4.4
Co-Cr-Mo	240	700	450	8	8.5
316 L steel	200	965	690	20	7.9
Cortical Bone	18	140	n/a	1	0.7
Dentine	18.3	52	n/a	0	2.2
Enamel	84	10	n/a	0	3

The mechanical properties of implant materials are list in Table 2.3 Strength is an important property because a high strength material better resists occlusal forces without fracture or failure. Lower modulus is also desirable because the implant biomaterial better transmits forces to the bone. All of six materials (cp titanium grade 1-4, Ti-6Al-4V and Ti-6Al-4V ELI) are commercially available. The implant selection depends on the individual patient. If a patient has a history of parafunctional habits and implant fracture, for example, the clinician should choose an implant made of titanium alloy, rather than cp grade I titanium. In additional, small-diameter implants indicate the need for higher-strength materials. Both elastic modulus and strength are important properties in choosing an implant material. The implant must have sufficient strength to withstand occlusal forces without permanent deformation, but should also have a low modulus for optimum force transfer [1].

There are many controversies about the toxicity of the alloy material. Some studies showed negative results from titanium, aluminium and vanadium ions (Ti^{4+}, Al^{3+} and V^{5+}) [28-30]. On the other hand, some studies showed no significant differences between cp Ti and Ti-6Al-4V [1-3, 31-33]. However, the Ti-6Al-4V alloy still used in Europe and US in hip prosthesis as no conclusive toxicity problems have been confirmed [3].

Bone biology

Bone tissue is arranged in two macro-architectural forms trabecular (or cancellous, or spongy) and cortical (or compact) [34]. The volume density of bone matrix in cortical bone is about 80-90%, in trabecular bone is only 20-25%. Therefore, trabecular bone contributes much less to the primary stability [25].

Suzuki suggests [5] that the histologic evaluation in trabecular bone in animal model is advantage more than in cortical bone because the analysis of trabecular bone response is desirable owing to regions of lower bone density often requiring more time for osseointegration establishment. But on the other hand, it was believed that bone marrow contained abundance of precursor cells for osteoblast and rich vascularity that cancellous bone could remodel far more quickly than cortical bone [25, 34].

The principle mechanism of osseointegration process

"Osseointegration" was first described by Brånemark and co-workers. The term was first defined as direct contact (at the light microscope level) between living bone and implant. Osseointegration is also histologically defined in *Dorland's Illustrated Medical Dictionary* as the direct anchorage of an implant by the formation of bony tissue around the implant without the growth of fibrous tissue at the bone-implant interface. Osseointegration is a critical step for the clinical success of implant [35].

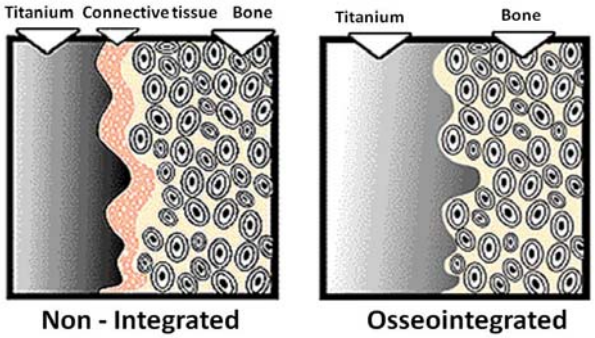

Figure 2.1 Drawings illustrate non-integrated compare osseointegrated to the implant surface.
Available from: http://www.wipp.se/komplett/possible/Pages/whatisoi.html [2012, January 18]

Phases of osseointegration [7, 25, 34, 36, 37]

The first healing phase of osseointegration is osteoinduction and osteoconduction. In the early bone response to the implant, blood will come into contact with the implant surface, with particular attention to platelets and fibrin (Figure 2.2 and 2.7). Proteins absorption comes from blood and tissue fluids at the implant site preparation and later the osteogenic cells recruit and migrate to the implant surface [38].

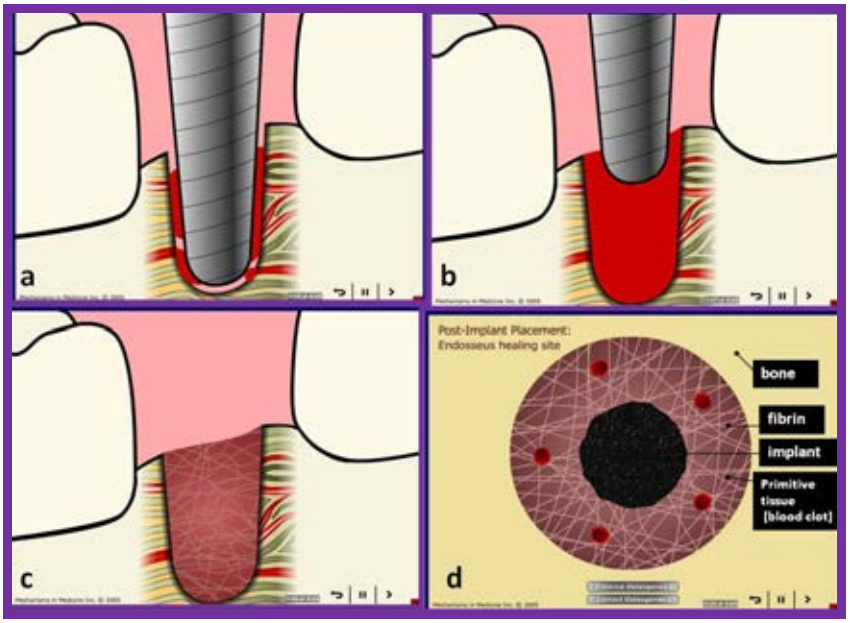

Figure 2.2 Drawings illustrate the early bone response to the dental implant. Available from: http://core-che.com/dentistry/visuals/dentistry_5.html [2012, January 15]

Red blood cells and the thrombogenic property are important to enhance coagulation and wound healing around implants for a successful bone formation on dental implant. The haemostatic with fibrin network on the titanium surface might be an optimal scaffold for regeneration of bone tissue [39, 40].

The implant surface affects to the retention of fibrin and the retention of fibrin also affects the new bone formation [34, 37]. Fibrin clot stabilization may play role in improved osseointegration. Effective fibrin retention shows in Figure 2.3 and inadequate fibrin retention shows in Figure 2.4.

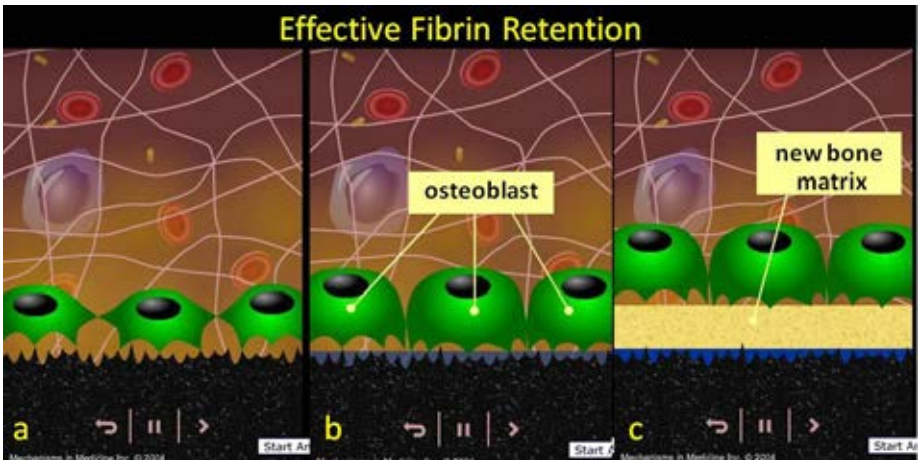

Figure 2.3 Drawings illustrate the effective fibrin retention. When the platelets are activated and released the growth factors, osteogenic cells migrate to the implant surface. In effective fibrin retention, the osteogenic cells can reach to the implant surface through fibrin. Finally, the bone cells secrete the bone matrix at the implant surface.
Available from: http://www.ecf.utoronto.ca/~bonehead/flash/retention.htm [2012, January 15]

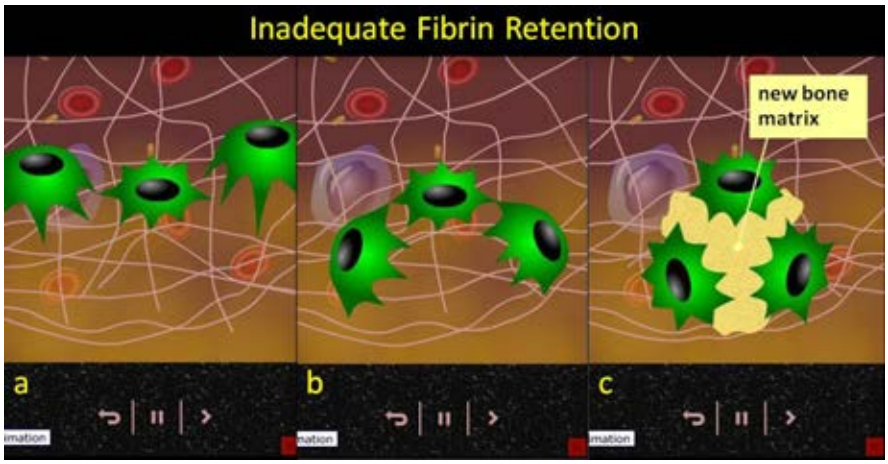

Figure 2.4 Drawings illustrate the inadequate fibrin retention. When the osteogenic cells migrated through the fibrin, they detach the fibrin from the implant surface. Also they stop migrating and begin liberate bone matrix. The result, the bone matrix would not be contact to the implant surface
Available from: http://www.ecf.utoronto.ca/~bonehead/flash/retention.htm [2012, January, 15]

The second healing phase of osseointegration, results in a mineralized. Interface matrix is the same as that seen in the cement line in natural bone. The peri-implant osteogenesis can proceed from the host bone to the implant surface (distant osteogenesis) and from the implant surface to the host bone (contact osteogenesis) in the so called *de novo* bone formation [34, 36, 37].

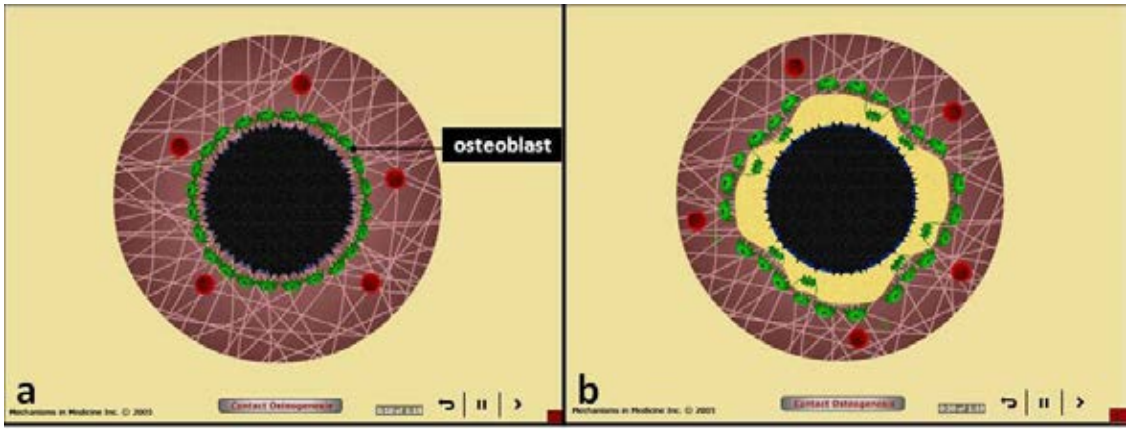

Figure 2.5 Drawings illustrate the stages of contact osteogenesis. The new bone is formed on the implant surface to the host bone.
Available from: http://core-che.com/dentistry/visuals/osteoCont.swf [2012, January 15]

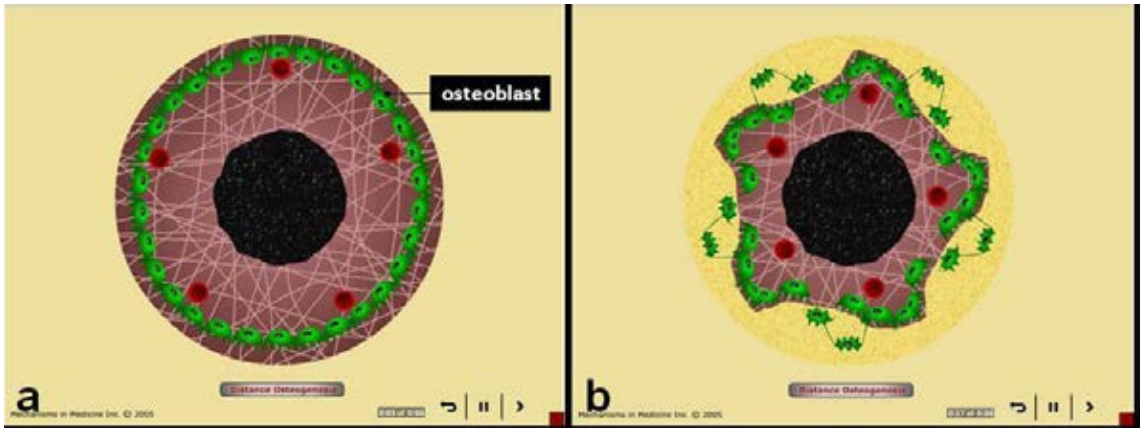

Figure 2.6 Drawings illustrate the stages of distance osteogenesis. The new bone is formed from the host bone to the implant surface.
Available from: http://core-che.com/dentistry/visuals/osteoDist.swf [2012, January 15]

The third healing phase of osseointegration, the long term remodeling of the tissue, is influenced by different stimuli [7]. The bone remodeling continues throughout life and thus becomes important for the longevity of implants [25].

Osseointegration is not an isolated phenomenon, but instead depends on previous osteoinduction and osteoconduction [35].

"Osteoinduction" means that primitive, undifferentiated and pluripotent cells are somehow stimulated to develop into the bone-forming cell lineage, i.e. the recruitment of immature cells and the stimulation of these cells to develop into preosteoblast, is a basic biological mechanical mechanism that occurs regularly, e.g. in fracture healing and implant incorporation [35].

"Osteoconduction" means that bone grows on a surface. An osteoconductive surface is one that permits bone growth on its surface or down into pores, channels or pipes. Bone conduction is not only dependent on conditions for bone repair, but also on the **"*biomaterial used and its reaction*"** [35].

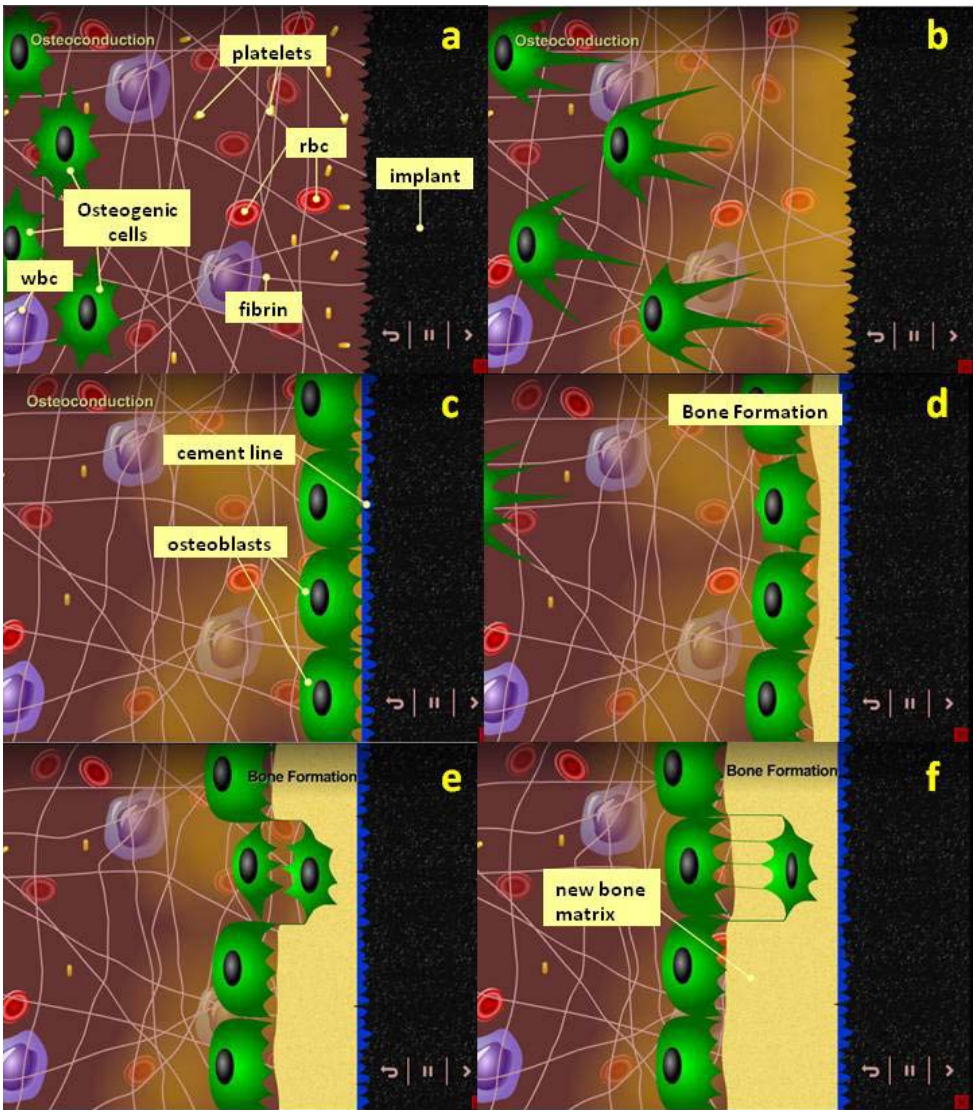

Figure 2.7 Drawings show the stages of **osteoconduction.** Osteoconduction is the key to contact osteogenesis. In a & b show the first healing phase. After drilling the implant site preparation, there are red blood cells, platelet (yellow particles), white blood cells and osteogenic cells at the wound site (a). When the platelets contact to the implant surface, they release some of the growth factors. The later, the osteogenic cells migrate to the implant surface (b). In c & d show the second healing phase. The osteogenic cells stop and grow on further they change the shape become osteoblast cells and secrete bone matrix formation on the implant surface (contact osteogesis). In e & f show the third healing phase, bone remodeling. The osteoblast cells continue activity, lie down, secrete bone matrix and mineralize. Some cells maybe embedded in the matrix and change to the osteocyte cells.
Available from: http://www.ecf.utoronto.ca/~bonehead/flash/osteoconduction.htm [2012, January 15]

Osseointegration factors

Many factors affect the bone-implant responses. Bone quantity, bone quality and site of bone are important from host [34, 38, 41]. These may vary in each patient. The cp titanium surface could be modified to enhance bone accrual suggested that cp titanium was not only “bioinert” or “biocompatibility”, but could influence cellular activity or tissue responses leading to greater osteogenesis [27].

Implant characteristics, such as type of materials [23, 38, 41], shape [36, 42], design [34, 36, 41, 42], implant surface properties [23, 34, 36, 38, 41, 43] (roughness, wettability, surface energy, surface chemistry, surface charged) influence the cells response. Furthermore, surgical techniques [23, 36, 38, 41] (skill of the surgeon, drilling with cooling agent), time and implant loading conditions [36, 38, 41] have been reported affecting the osseointegration. Therefore, implant biocompatibility and ability to osseointegrate can be modified by various factors.

Roughness

Rough surfaces have been proposed to enlarge the material area in contact the cells response [36]. The osteoblast-liked cells show a tendency to attach more quickly and with more differentiated faster on rough surfaces than the smooth surfaces [6]. The rough implant surface accelerated the osteoblastic gene expressions and improved adhesive bone-implant strength compare with the smooth surface [25].

Albrektsson and co-worker [44] reviewed roughness of dental implant and classified the roughness in 4 classes (show in Table 2.4). The studies showed that implants with moderate surface roughness (1-2 μm) have a stronger bone response compared with a smoother or rougher surface [44-47].

Table 2.4 Classification degree of dental implant roughness by Albrektsson [44]

Classification	Roughness (S_a)
Smooth	0.0-0.4 μm
Minimally rough	0.5-1.0 μm
Moderately rough	1.0-2.0 μm
Rough	>2.0 μm

There is currently considerable interest in nanostructures. Positive bone responses occur at nanostructured surfaces tested *in vitro* and *in vivo* [27]. However, biomaterials interfaces at nanoscale will be defined by long-term clinical study [27, 44].

Hydrophilicity / Wettability

The wettability effect to the absorption of proteins onto material surface, cell adhesion on hydrophilic surface is better than on hydrophobic surface [15, 27].

The contact angle is one of the techniques that evaluate the hydrophilicity of the surface. The contact angle is the angle at which a liquid/vapor interface meets a solid surface. The classification of contact angle was illustrated in the Table 2.5.

Table 2.5 Classification of hydrophilicity

Classification	Contact angle
Highly hydrophilic surface	0° to 30°
Hydrophilic surface	>30° to 90°
Hydrophobic surface	>90° to 120°
Highly hydrophobic surface	>120° to 150°
Super hydrophobic surface	>150°

Available from: http://en.wikipedia.org/wiki/Contact_angle [2011, February 18]

Surface energy

Hydrophilic surface has high surface energy. A surface with a high energy has a high affinity for protein adsorption and shows stronger osseointegration than implants with a low surface energy. However, the study on implants with a high surface energy results in stronger osseointegration has not been verified *in vivo* studies [44].

Surface charge

Surface charge has been reported to affect the absorption of proteins onto material surface [44]. Implant products from many companies have charge on the implant surface such as SLA & SLActive (Straumann), Osseospeed (fluoride ion; Astra Tech). Evidence suggests that the surface charge of the hydrophilic SLActive surface may selectively attract proteins surface such as hat which exert specific up- or down-regulations of genes expressed by the adjacent progenitor cells [48]. However, both of negatively and positively charged surfaces were observed to promote bone formation [38, 49].

Chemical properties / surface chemistry

The chemical composition of the surface can affect to cell-material interaction and can also promote osseointegration. There are many chemicals and

techniques to modify cp titanium to be a bioactive material such as collagen, RGD, chitosan, BMP (bone morphogenetic proteins), fluoride, calcium phosphate or hydroxyapatite (HA), NaOH and heat treatment, ion implantation with calcium, or anodizing with electrolytes containing phosphorus, sulphur, calcium, or magnesium ions [44].

Surface modification techniques of dental implants

Implant properties have been developed in the last decade in a concentrated effort to provide bone with the faster and improved osseointegration process for decreasing the treatment periods between implant placement and restoration [4, 12, 23, 50]. Today, a growing aspect of endosseous implant surface research is focused on further enchancing the activity of bone forming cells at the tissue implant interface. The desire for “bioactivity” has been addressed using a variety of different approaches [27].

There are many techniques to alter the implant surface properties such as modification of the surface roughness by mechanical blasting [4-6] and acid etching [4-6], or chemical modification by plasma-sprayed hydroxyapatite [4, 7] and bioceramic coating [5] or Ca P deposition [7], or coating with extra cellular bone matrix component (collagen [8, 9], RGD peptide [9-11], chitosan [12], etc.), or forming oxide layers by electrochemical anodization [13-15] and sol-gel [16], or polyelectrolyte multilayer (PEM) films technique [17-19].

However, weak interfaces between the coating and implant surface, such as those found in plasma-sprayed hydroxyapatite and the potential high susceptibility to bacterial colonization and peri-implant tissue disease, raised concerns with respect to their long term clinical performance [4, 5, 12, 23].

Increasing surface roughness by etching, plasma spraying, sintering, and/or sandblasting is used to increase contact area between implant and host bone for enchanced biomechanical locking, stability, and peri-implant bone formation [12, 47]. However, sandblasting with nonresorbable materials may leave residual particles embedded in the implant surface, which may lead to inflammatory responses and inhibit integration [12].

Nowadays, the best technique has yet not declared. The new techniques are under continuously developed.

Polyelectrolyte multilayer films (PEMs)

Polyelectrolytes are polymers that contain relatively high degree of ionizable groups along their backbone chains. Polyelectrolytes can be cationic, anionic or amphophilic (contain both cationic and anionic groups that are present in the *same* or *different* monomer units). Polyelectrolytes can be synthesized by polymerization of monomer units or by modification of the polymer to induce charges on the monomer repeating units.

The build-up of polyelectrolyte multilayer films (PEMs) is now widely used for the modification of biomaterial surfaces in clinical applications as implant materials, prosthesis, and facial organs in order to enhance their bioactive through the adsorption of protein or other biological molecules capable of transmitting signals to contacting cells. The principles are based on a layer-by-layer method (LbL) and assembly of positively and negatively charged polyelectrolytes, resulting in multilayer films. This technique is of particular interest in dental and orthopedic implantology for tissue engineering and surface functionalization [18, 20].

For about 70 years, the molecularly controlled fabrication of nanostructured films has been dominated by the so-called **Langmuir-Blodgett (LB) technique**, in which monolayers are formed on a water surface and then transferred onto a solid support. But the LB technique requires special equipment and has severe limitations with respects to substrate size and topology as well as film quality and stability [21].

Since the early 1980s, **self assembly techniques** were developed as an alternative to LB films. However, self assembled films based on covalent or coordination chemistry are restricted to certain classes of organics, and high-quality multilayer films cannot be reliably obtained [21].

Since the early 1990s, Decher [21] has developed **polyelectrolyte multilayer technique** (PEM). The principle is based on an electrostatic layer-by-layer (LbL) self-assembly process. PEMs films were constructed by alternated adsorption of polyanions and polycations at the surface of materials, which can be easily obtained by simple dipping in polyelectrolyte solutions. The electrostatic attraction between oppositely charged molecules seemed to be a good candidate as a driving force for multilayer build-up. The multilayer built by the LbL method offered a more stable coating than that prepared by physical adsorption because of the electrostatic attractions between layer to layer and layer to substrate. The process, which is extremely simple, is depicted in Figure 2.8.

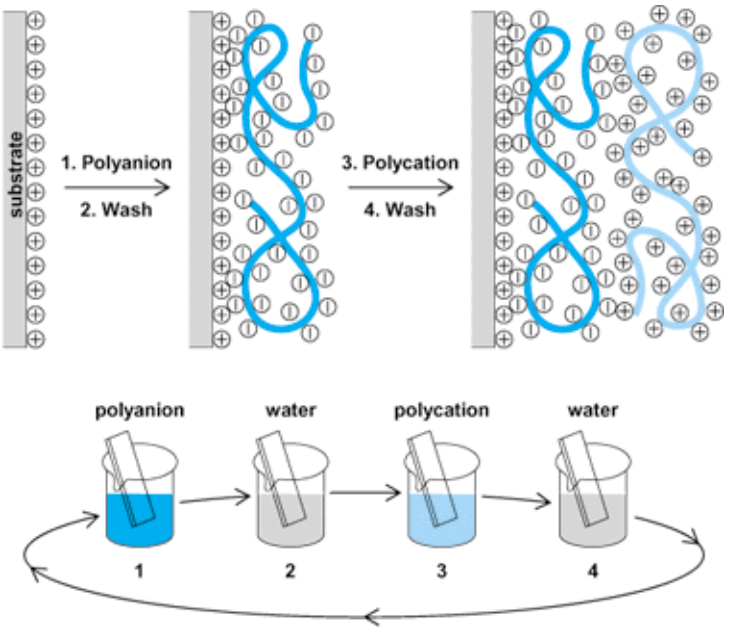

Figure 2.8 The polyelectrolyte multilayers films process.

Available from: http://www.google.co.th/imgres?q=The+polyelectrolyte+multilayers+films+process&hl=th&biw=1366&bih=667&gbv=2&tbm=isch&tbnid=oKzUcM-eXnLkMM:&imgrefurl=http://accessscience. [2012, February 22]

The process begins by properly charging a substrate. The charged substrate is dipped into the first oppositely charged polyelectrolyte solution for a certain period of time to allow the polyelectrolyte to adsorb to the surface. After being exposed to the oppositely charged polymer, the surface is then immersed in a rinse solution to wash off the loosely bound polymer as well as to prevent cross-contamination of the polyelectrolyte solutions. The substrate is then dipped into a polyelectrolyte solution of opposite charge. This second polyelectrolyte adsorbs to the surface due to electrostatic attraction and actually overcompensates for the surface charge resulting in a reversal of the surface charge. These simple steps complete the LbL deposition of the nanolayers. The substrate may be immersed and rinsed, in an alternating fashion, in the two polyelectrolyte solutions to form the multilayer layers. The process is repeated until the desired number of layers is achieved. Each step results in a reversal of surface charge allowing the next layer to be deposited [21].

The advantages of layer-by-layer adsorption from solution are that many different materials can be incorporated in individual multilayer films and, versatile, inexpensive, yet efficient technique to build biologically active surfaces for multiple purposes [17, 21].

These new biocompatible coatings for implants requires the control of several physio-chemimal parameters such as layer assembly sequence, thickness, surface charge, pH changes, salt concentration of polymer, roughness, biodegradability, biomechanical properties, and more importantly biocompatibility [18]. However, Wittmer *et.al.* [20] found film (polymer) composition, terminal layer, and rigidity to be the most important properties in promoting cell attachment, growth, and function.

Many polyelectrolytes, such as poly(ethyleneimine) (PEI), Poly(styrene sulfonate) (PSS), and poly(allylamine hydrochloride) (PAH), chitosan (Chi), gelatin (Gel), can be successfully deposited onto titanium. The PEM films can improve cells growth on titanium surfaces [17, 18].

Recently, Angwarawong *et al*. [22] has developed {$(PDADMAC/PSS)_4$ PDADMAC} PSS-co-MA PEM films on glass surfaces, it is successful to promote the osteoblast cell (MC3T3-E1) function over the control glass.

Poly (diallyldimethylammonium chloride) **(PDADMAC**) is strong cationic polyelectrolyte which consist of positive charges along the backbone chain. In contrast, Poly (sodium 4-styrene sulfonate) **(PSS**) is strong anionic polyelectrolyte which consist of negative charges along the backbone chain. Both PDADMAC and PSS were used in the study to investigate factors influencing for the properties and structure of polyelectrolyte multilayer films [51].

Poly(4-styrenesulfonic acid-co-maleic acid) sodium salt **(PSS-co-MA)** is a copolymer of PSS and maleic acid (MA). The ionization depends on the pH. At high pH, PSS-co-MA was used as anionic polyelectrolyte which included strong anionic group (sulfonate group) and weak anionic group (carboxylic group). Building a multilayer film with copolymer consisting of both weak and strong polyelectrolyte pendant groups may obviate the need for chemical cross-linking to improve the stability of weak polyelectrolyte multilayers. In such a case, the strongly charged groups can form electrostatic linkages (thereby enhancing film stability), while the weakly charged groups can be used to alter multilayer properties because they are responsive to external pH changes [52].

{$(PDADMAC/PSS)_4$/PDADMAC}PSS-co-MA PEM films coated on glass surfaces were examined on its ability in affecting osteoblast functions. Although, no differences were observed in MC3T3-E1 in cell attachment or spreading on either PSS-co-MA PEM films or glass at 4-16 hours, but PSS-co-MA PEM films can promote ALP activity at day-7, the expression of OPN, BSP and OC at day-13. Moreover, cells cultured on PSS-co-MA film developed faster rate of in vitro calcium deposition at day-15 compared to the control *in vitro* [22].

Therefore, it is interesting to investigate the cell response to PSS-co-MA PEM films when coated on the other materials, such as titanium surface. In this study, we used osteoblast cell line (MC3T3-E1) grown on the cp titanium disc (grade 2) coated with {(PDADMAC/PSS)$_4$/PDADMAC}PSS-co-MA PEM films compare to uncoated titanium surface to examine the bone formation in the *vitro* study and to evaluate the bone formation of {(PDADMAC/PSS)$_4$/PDADMAC}PSS-co-MA PEM films on cp titanium pin (grade 2) compare to the uncoated titanium on the rat model.

CHAPTER III

RESEARCH METHODOLOGY

Titanium discs and pins preparation

Titanium (Ti) rod and Ti wire were obtained from commercially pure titanium grade 2 (KVM Heating Element Co.,Ltd., Bangkok, Thailand). The Ti disc was prepared from 15-mm. Ti-rod. The rod was cut to 3-mm. thickness disc and polished using 400, 800, 1000-grit SiC paper in a polishing machine (DPS 3200, IMPTECH, South Africa). Ti wire, 1 mm. in diameter, was cut to 8 mm. in length. All samples were ultrasonically cleaned with 10 min in acetone followed by 10 min in ethanol, rinsed with de-ionized water and air dried before dipping. The Ti discs were used in the *vitro* study and the Ti pins were used in the *vivo* study.

Fabrication of polyelectrolyte multilayer films on titanium

{(PDADMAC/PSS)$_4$/PDADMAC}PSS-co-MA polyelectrolyte multilayer (PEM) films were constructed by forming 9 layers of poly(diallyldimethylammonium chloride) (PDADMAC) and poly(sodium 4-styrene sulfonate) (PSS) with a stop layer of poly(4-styrenesulfonic acid-co-maleic acid) sodium salt (PSS-co-MA) (All chemicals were obtained from Sigma-Aldrich, Germany, Figure 3.1). Briefly, Ti discs were alternatively immersed in 10mM PDADMAC in 0.1M NaCl or 10mM PSS in 0.1M NaCl for 5 min, respectively, with intermediate triple rinses with distilled water until the ninth layer were formed. For the final layer, the Ti discs were immersed in 10mM PSS-co-MA (pH10) in 0.1M NaCl for 30 min, rinsed with distilled water (pH 10) and steriled under the laminar hood by washing with 70% ethanol for 10 min, three times rinsing with de-ionized water and air dried [22]. The fabrication method diagram is shown in Figure 3.2.

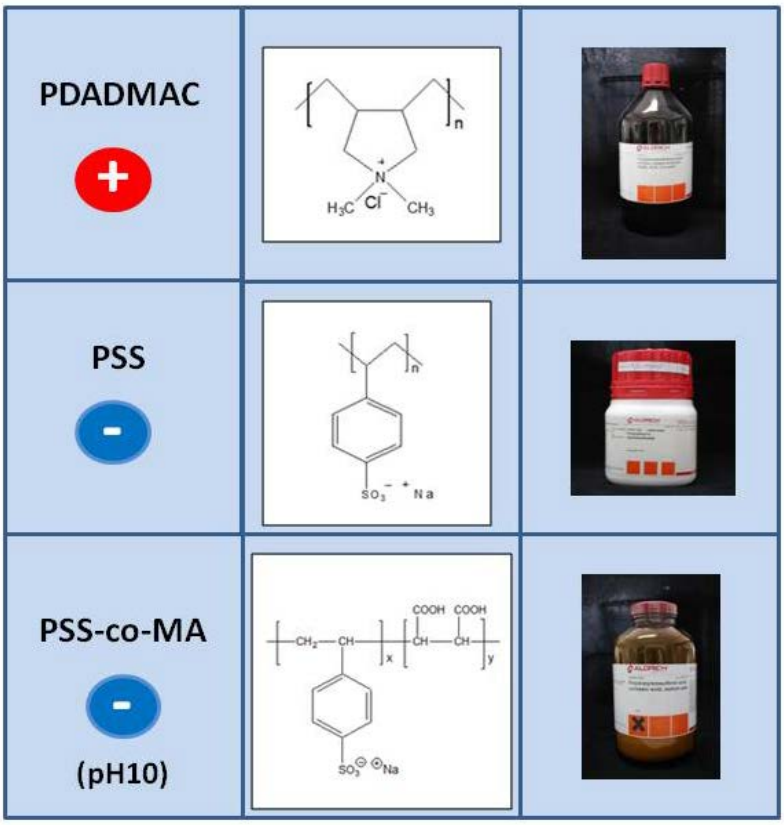

Figure 3.1 Chemicals used in this research

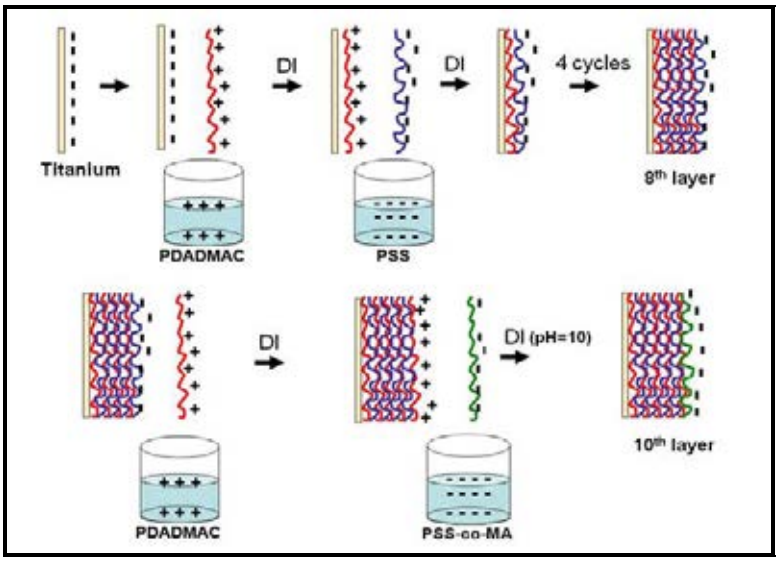

Figure 3.2 Scheme of fabrication of {(PDADMAC/PSS)$_4$/PDADMAC}PSS-co-MA PEM films
Modified from: Angwarawong T. Modification of titanium surface for supporting osteoblast adhesion and differentiation. Doctoral dissertation, Philosophy Program in Oral Biology Faculty of Dentistry Chulalongkorn University. 2011

Surface characterization analysis

- ***Surface morphology***

Surface morphology of the Ti discs was examined by a scanning electron microscope (SEM) (JSM 5410LV, JEOL, Japan) at magnification x 1,500.

- ***Surface roughness***

Surface roughness was measured by atomic force spectroscopy (AFM; Nanoscope IV, Multimode, Veeco, Santa Barbara, CA, USA). Average surface roughness (R_a) and the root mean square roughness (R_q) were calculated from three independent samples.

- ***Hydrophilicity***

Hydrophilicity was determined by measure the static contact angle measurement using Krüss (model DSA 10, Hamburg, Germany) at ambient temperature. The measurement was performed by dropped a 10 µl sessile droplet of de-ionized water vertically on the specimen surface without physical contact using a micro-syringe onto the film surface. The contact angles were measured ten times and then averaged.

Fibrin clot formation

The protocol was approved by the Ethical Committee, Faculty of Dentistry, Chulalongkorn University. Whole blood was collected from the healthy volunteer (30 years old, n=1). The 150 µl of the whole blood, without any addition of anticoagulants, was dropped on the Ti surfaces and then covered with glass cover slips immediately. After 5 min, the specimens were rinsed in 0.1M PBS. They were dehydrated in a graded series of ethanol (30%, 50%, 70%, 90% & 100%), 2 min in each concentration and then critical point dried with 100% hexamethyldisilazane (HMDS, Fluka, Steinheim, Germany) for 5 min. Gold was sputter-coated on the surface and the samples were examined using scanning electron microscope (JSM 5410LV, JEOL, Japan) at magnification x1,500 and x3,000. The experiments were performed in triplicate.

Osteoblast cells interaction on titanium discs

Cells culture

MC3T3-E1 cells (ATCC CRL-2593), the mouse osteoblast cell line, were seeded on coated and uncoated Ti discs at density of 50,000 cells per well in osteogenic medium (HyQ® MEM/EBSS, HyClone, Logan, Utah, USA) supplemented with 10%

fetal bovine serum (FBS, ICP biologicals, Henderson, Auckland, New Zeland), 2 mM L-glutamine, 100 unit ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 0.25 µg ml^{-1} amphotericin B (Gibco,Grand Island, New York, USA), 5 mM glycerol-2-phosphate disodium salt hydrate (β-glycerophosphate; Sigma, St. Louis, MO, USA) and 50 µg ml^{-1} l-ascorbic acid sodium salt (Sigma, St. Louis, MO, USA) under standard condition (at 37°C in 100% humidity and 5% CO_2). Cell from passage 18 to 25 were used in the experiments. The medium was changed every other day.

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

Expressions of type I collagen (Col I), osteopontin(OPN), bone sialoprotein (BSP), and osteocalcin (OC) messenger RNA (mRNA) were assessed using qRT-PCR. Total RNA was extracted with TriPure Isolation Reagent (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacture protocol. First strand DNA was reverse transcribed from 1µg of total RNA using reverse transcriptase enzyme (ImProm-II Reverse 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 45 cycles of 95 °C for 10 S; 60 °C for 10 S: 72 °C for 25 S. PCR oligonucleotide sequences of the primers are shown in the Table 3.1. 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). The house keeping gene, GAPDH, was used as a reference control. The expression ratios for gene were performed using the Roche LightCycler 480 software version 1.5 (Roche, Mannheim, Germany). The experiments were performed in duplicate.

Table 3.1 Primer sequences for qRT-PCR analysis

Gene name	Sense	Antisense
GAPDH	5'ACTTTGTCAAGCTCATTTCC3'	5'TGCAGCGAACTTTATTGATG3'
Col I	5'GGTGCCCCCGGTCTTCAG3'	5'AGGGCCAGGGGGTCCAGCATTTC3'
OPN	5'CCAACGGCCGAGGTGATA3'	5'CAGGCTGGCTTTGGAACTTG3'
BSP	5'TGTCTGCTGAAACCCGTTC3'	5'GGGGTCTTTAAGTACCGGC3'
OC	5'CTTGGGTTCTGACTGGGTGT3'	5'AGGGAGGATCAAGTCCCG3'

Alizarin red-S staining

In vitro mineralization was quantified by Alizarin red-S staining (Alizarin Red S –certified, Sigma, St.Louis, MO, USA) after 15 days of cells culture. Cells were fixed

with cold methanol for 20 min and stained with 1% Alizarin red in 1:100 (v/v) ammonium hydroxide/water (pH 4.2) for 3 min. The amount of calcium deposition was quantified by destained with 10% cetylpyridinium chloride monohydrate (Sigma, St. Louis, MO, USA) in 10mM sodium phosphate at room temperature for 15 min. The absorbance was measured at 570 nm using the UV-vis spectrophotometer. The experiments were performed in triplicate. Results are shown means of each group.

Evaluate the bone formation on titanium pins *in vivo*

Animals, anesthesia and surgical technique

Eight male Wistar rats *(Rattus norvegicus)* average 10 weeks old (250-300 gm body weight), obtained from National Laboratory Animal Centre, Mahidol University, were used. The rats were anesthetized by intraperitoneal injections of 50mg/ml sodium pentobarbital (60 mg/kg body weight). One coated Ti pin was placed in one femur while an uncoated pin was placed into the other. A total of 8 uncoated titanium pins and 8 coated titanium pins were used in 8 rats. The drill was made using a steel dental bur no.010 with continuous external saline cooling and then implanted the pin into the medullary canal of the femur. The wound was closed using resorbable vicryl 5-0 sutures (Ethicon, Johnson & Johnson, Belgium). Housing and feeding of the animals was according to standard animal care protocols. The protocol was approved by the Animal Care and Use Ethical Committee, Faculty of Dentistry, Chulalongkorn University. Four rats were sacrificed at 2 weeks and the others at 4 weeks.

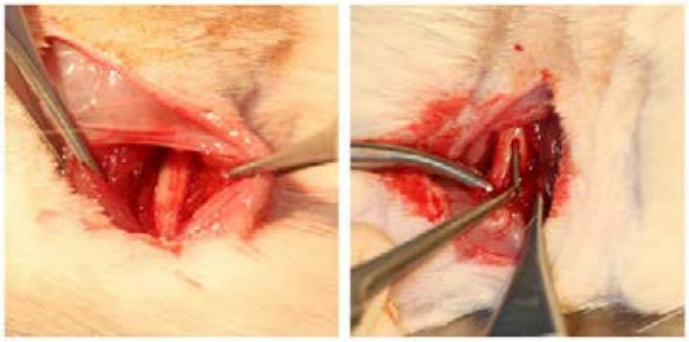

Figure 3.3 Titanium pin was implanted into the femur of the rat.

Histological examination

After sacrificed, the femurs of rats were removed and radiography (Figure 3.4) examined to locate the implant location. The femurs were cut with diamond saw to obtain a suitable size sample, fixed in 4% formaldehyde, dehydrate in a series of ethanol (30%, 50%, 70%, 90% & 100%) and embedded in 100% Micro-bed resin (EMS, Hatfield, PA, USA) (Figure 3.5). One hundred µm undecalcified ground cross

sections were prepared using a sawing microtome technique (Leica, SP1600, Nussboch, Germany, showed in Figure 3.6). The sections were stained with alizarin red and analyzed histomorphometrically with computer software.

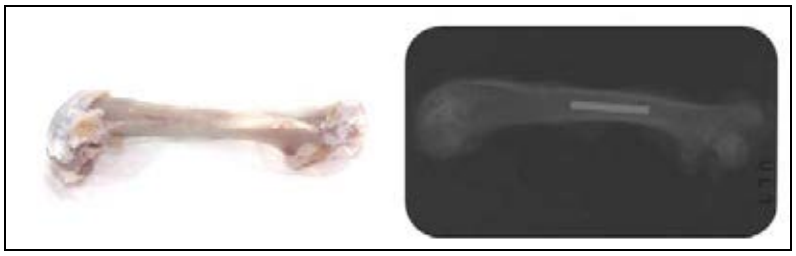

Figure 3.4 Radiography examination

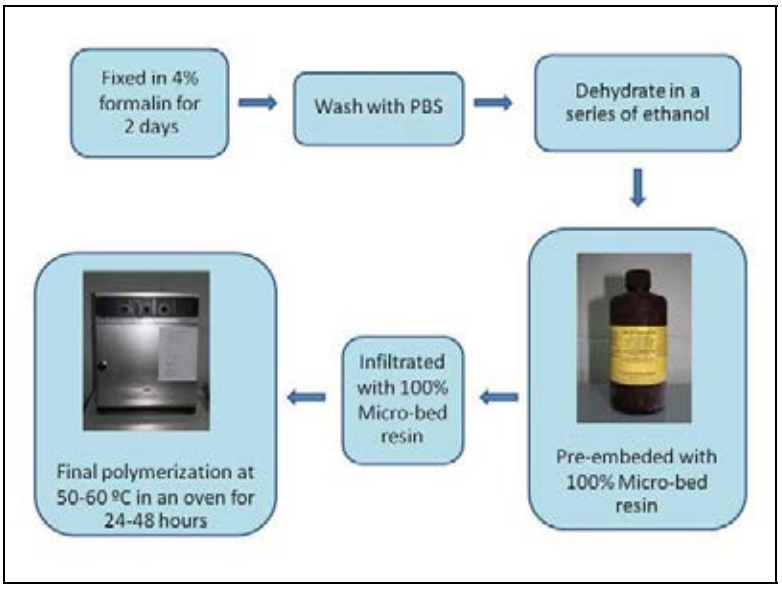

Figure 3.5 Scheme of embedding the sample in resin

Figure 3.6 Saw microtome

The new bone formations were analyzed histomorphometrically in two terms. The direct contact of new bone circumference at the Ti surface, in term of Bone-to-implant contact (BIC), was calculated as a percentage of the total Ti circumference by AxioVision 4.8.1 software (Carl Zeiss, Microimaging GmbH, Germany). Moreover, the amount of new bone formation around the Ti surface, in term of Bone volume (BV), was reported by detecting the total bone content within a circle of 0.1 mm around the Ti and calculated as a percentage by Image-Pro® Plus software, Version 6.0, Media Cybernetics, Inc., USA. (Figure 3.7) The examiner was blinded and calibrated before study.

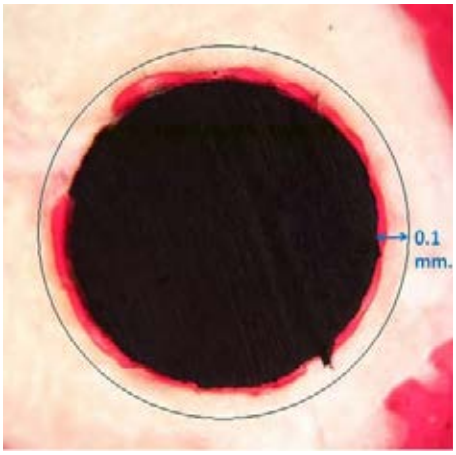

Figure 3.7 The percentage of bone volume was calculated the new bone formations within 0.1 mm. around the titanium surface. The newly bone formed around the pin showed in the red color.

Statistical analysis

Normal distribution of the data was confirmed and then t-test or Mann Whitney U test (Mann Whitney U test was used only for qRT-PCR) was used to compare mean between the coated PSS-co-MA PEM films titanium group and the uncoated titanium group. The data were presented as the mean ± SD. The significant was considered at $p < .05$ SPSS® 17.0 software (SPSS, Chicago, IL, USA) was used for all analysis.

CHAPTER IV

RESULTS

Surface characteristics of titanium discs

Surface morphology and Surface roughness

The results of surface morphology as analyzed by SEM and AFM were shown in Figure 4.1 and Table 4.1. No obviously difference was detected by SEM pictures.

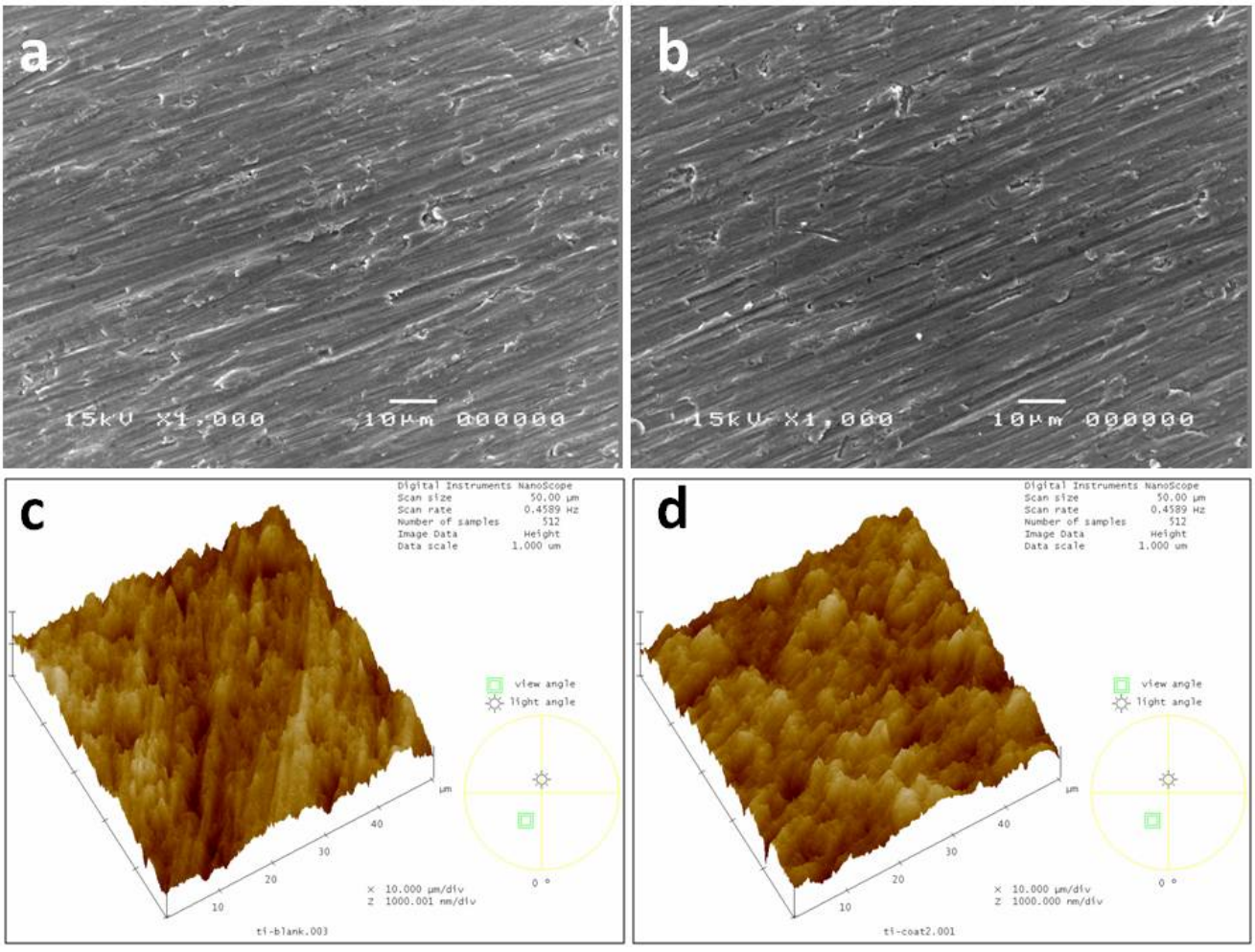

Figure 4.1 Surface morphology (*a:* uncoated Ti disc, *b:* PSS-co-MA coated Ti disc) analyzed by SEM (at magnification x 1,000) and surface roughness (*c:* uncoated Ti disc, *d:* PSS-co-MA coated Ti disc) analyzed by AFM.

Table 4.1 Surface roughness determination of uncoated Ti surface and PSS-co-MA coated Ti surface by AFM.

Materials	Ra (nm)	Rq (nm)
Ti_control	148.92 ± 8.73	188.38 ± 11.69
Ti_PSS-co-MA	142.70 ± 8.26	182.35 ± 7.84

Similarly, results from AFM in Table 4.1 also showed the similar values of *R*a and *R*q. The *R*a of uncoated Ti disc surfaces and PSS-co-MA coated Ti disc surfaces were 148.92 ± 8.73 nm and 142.70 ± 8.26 nm, while the *R*q of uncoated and PSS-co-MA coated Ti disc surfaces were 188.38 ± 11.69 nm and 182.35 ± 7.84 nm, respectively. Statistical analysis revealed no significantly difference between uncoated Ti disc surfaces and PSS-co-MA coated Ti disc surfaces for both of *R*a and *R*q. (p>0.05)

Hydrophilicity

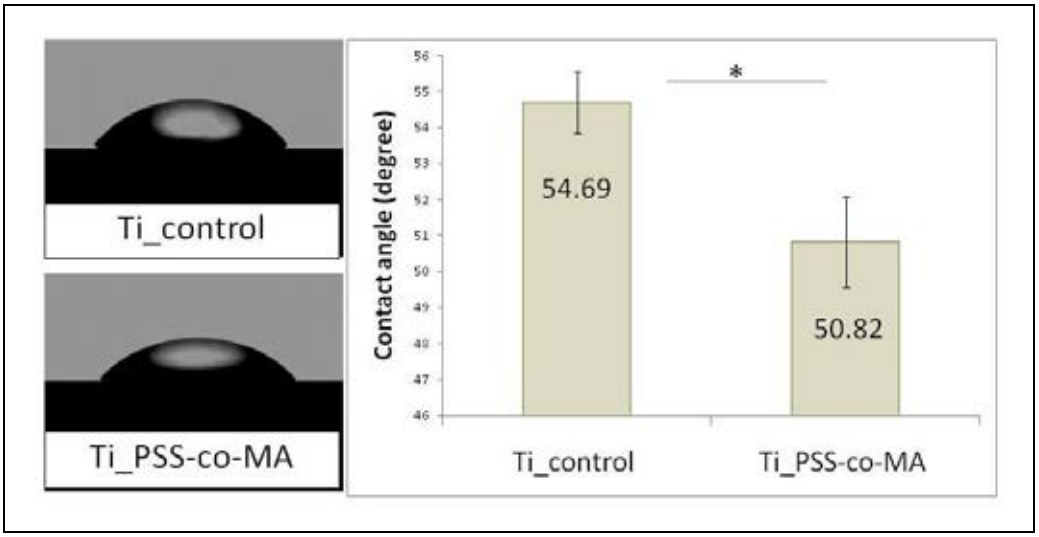

Figure 4.2 The wettability of the uncoated titanium surfaces and the PSS-co-MA coated titanium surfaces. (*Statistically significant, p<0.01)

Results in the Figure 4.2 showed that PSS-co-MA coated Ti surface had significant lower contact angle than the uncoated Ti disc. (p<0.01). The contact angle of uncoated Ti surface and PSS-co-MA coated Ti surface were 54.69 ± 0.86 degree and 50.82 ± 1.27 degree.

Fibrin clot formation

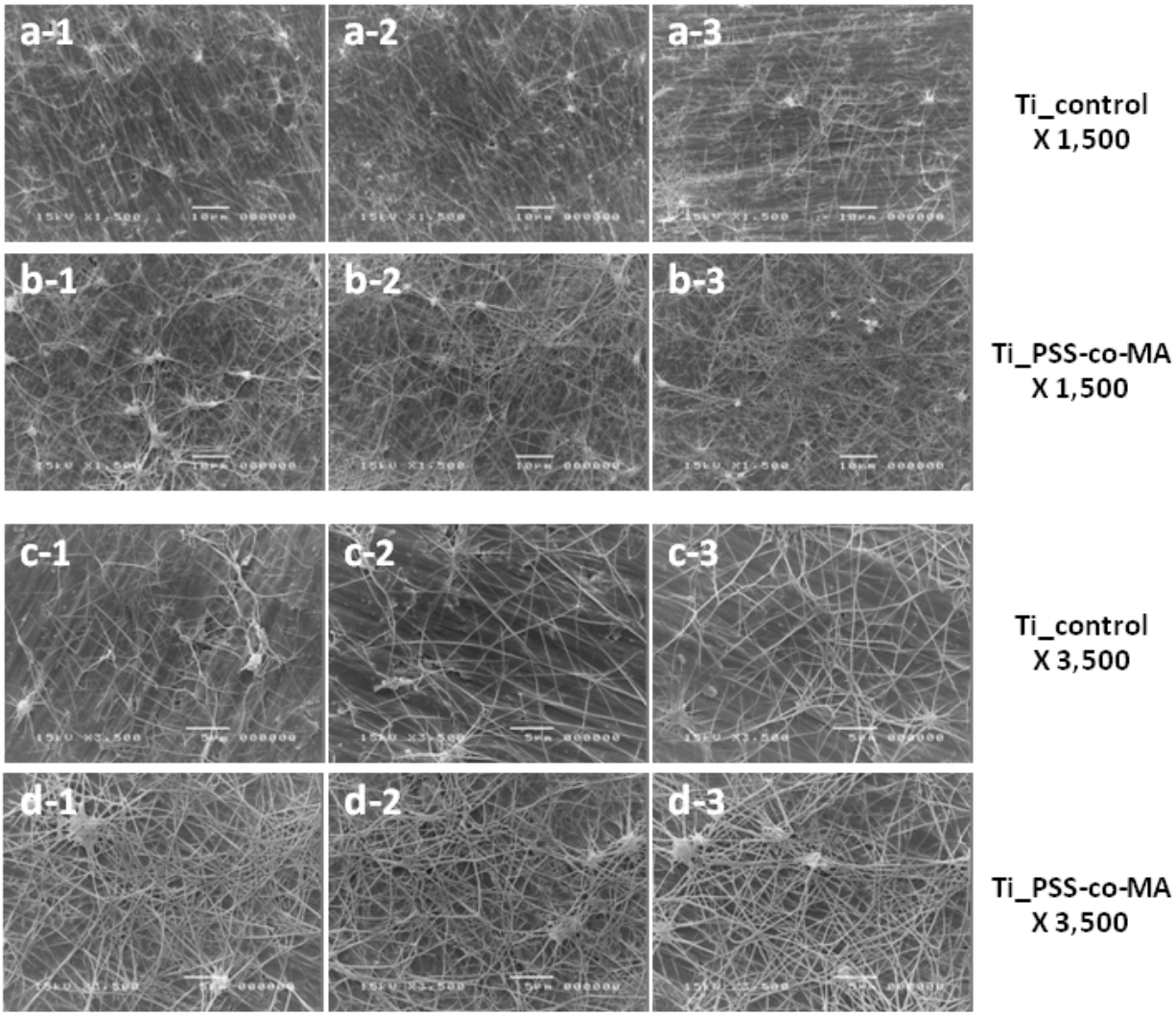

Figure 4.3 The fibrin formations on the titanium surfaces (n=3 of each group). *a(1-3) & c(1-3)*: uncoated Ti surfaces; *b(1-3) & d(1-3)*: PSS-co-MA Ti surfaces, at 5 min after dropping the whole blood analyzed by SEM at magnification x 1,500 (*a & b*) and x 3,500 (*c & d*).

The Figure 4.3 showed the appearance of the fibrins on the titanium disc surface after 5 min of blood exposure. The amount of fibrin formations on the PSS-co-MA coated Ti surface showed greater than the uncoated Ti surface.

Osteoblast cells interaction on titanium discs

Osteoblastic gene expression

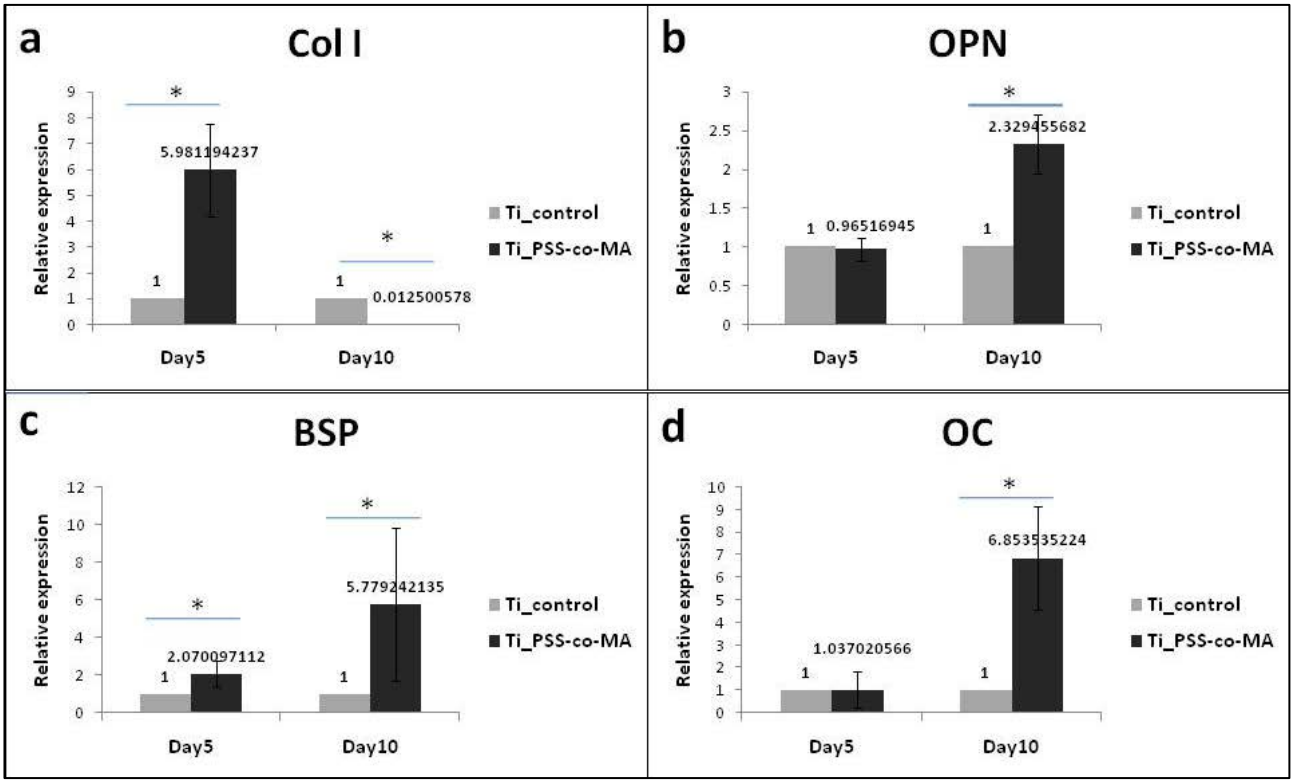

Figure 4.4 Osteoblastic gene expressions : the expression of Col I (a), OPN (b), BSP (c) and OC (d) in MC3T3-E1 cells cultured on uncoated Ti surface and coated PSS-co-MA Ti surface at day5 and day 10 were examined using qRT-PCR analysis. (*Statistically significant, $p<0.05$)

The expressions of osteoblastic related gene (Col-1, OPN, BSP, OC) were examined by qRT-PCR at day5 and 10 (Figure 4.4). Result showed the expressions of Col-1 were significant increased in cell cultured on PSS-co-MA coated Ti surfaces when compared to uncoated Ti discs at day5, but not at day10. The expressions of BSP were significantly increased in Ti coated surfaces both at day5 and day 10, while the expressions of OPN and OC increased only at day 10 on coated surfaces compared to those on the uncoated discs ($p<0.05$).

Alizarin red-S staining

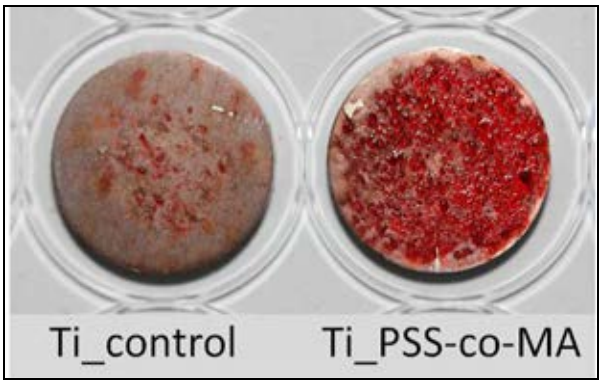

Figure 4.5 *In vitro* calcification on uncoated Ti disc and PSS-co-MA coated Ti disc at day15

Results from Alizarin red-S staining (Figure 4.5) showed stronger staining in cell cultured on PSS-co-MA coated Ti surfaces compared to the staining on uncoated Ti disc at day15.

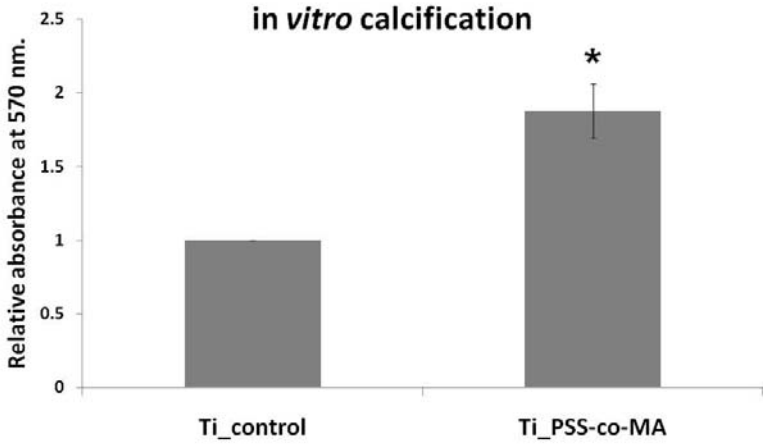

Figure 4.6 The amount of *in vitro* calcium deposition at day15 of uncoated Ti discs and PSS-co-MA coated Ti discs was quantified by destained with 10% cetylpyridinium chloride monohydrate and measured the absorbance at 570 nm. (*Statistically significant, $p<0.05$)

In vitro calcification at day 15, the amount of calcium deposition was quantified. The relative absorbance of PSS-co-MA coated Ti surface was significant greater than uncoated Ti discs ($p<0.05$).

Bone formation on titanium pins *in vivo*

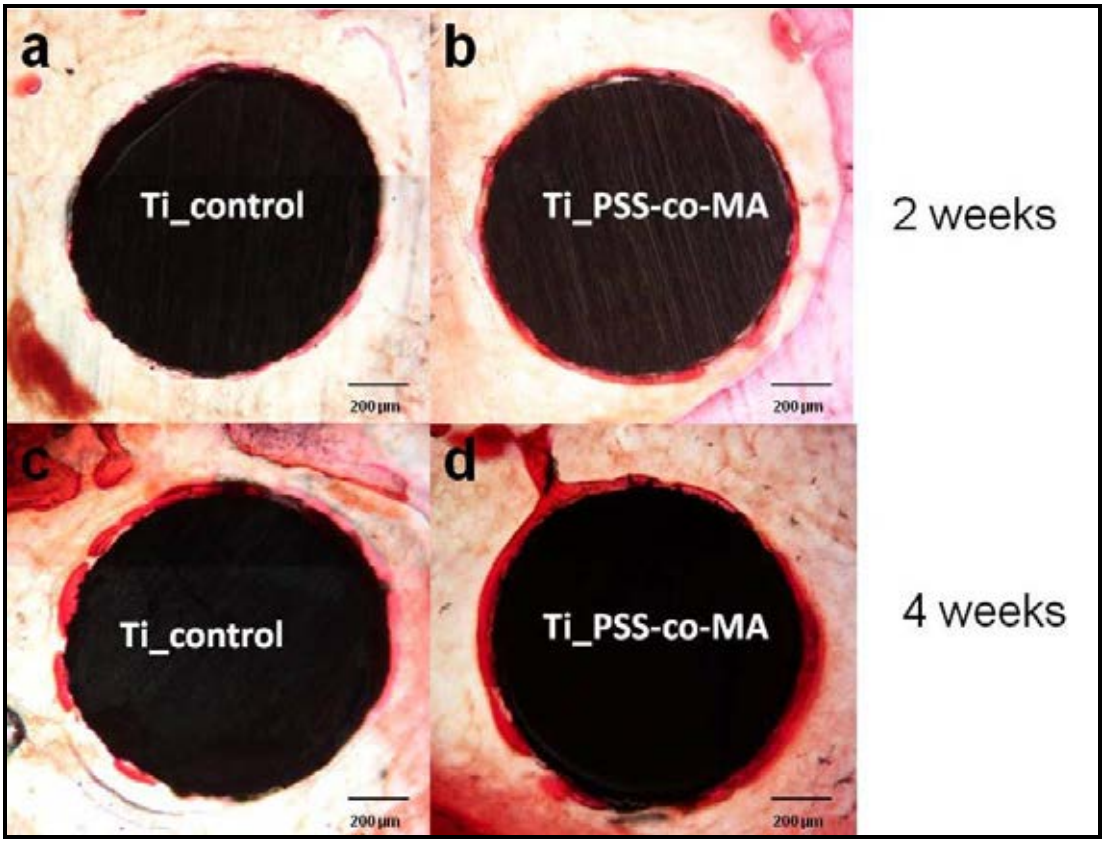

Figure 4.7 Bone formations around the uncoated Ti pins (a & c) and the PSS-co-MA coated Ti pins (b & d) at 2 weeks (a & b) and 4 weeks (c & d) when implanted in the rats, were determined by Alizarin red-S staining at magnification 10X.

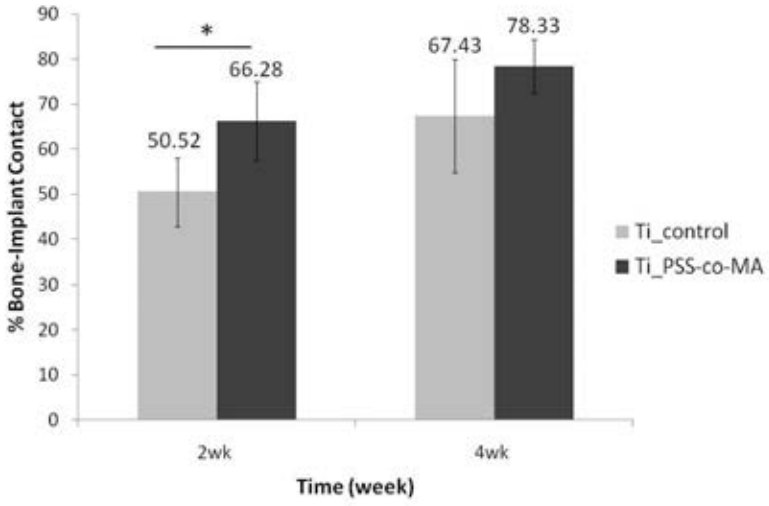

Figure 4.8 The percentages of bone-to-implant contact circumference the titanium pins. (*Statistically significant, p<0.05)

The results showed the presence of new bone formations around the Ti pins after implanted for 2 and 4 weeks (Figure 4.7). Histomorphometric analysis, shown as percentages of bone-to-implant contact (Figure 4.8), showed a significant increased in bone formations around PSS-co-MA coated Ti pins when compared to uncoated pins at 2 weeks (p<0.05). At 2 weeks the percentages of direct bone contact with the uncoated Ti pins and PSS-co-MA coated Ti pins surfaces were 50.52 ± 7.60 and 66.28 ± 8.74, respectively. However, at 4 weeks no significant difference at 4 weeks (p>0.05) was found. The percentages of direct bone contact at 4 weeks were 67.43 ± 12.62 in uncoated Ti pins and 78.33 ± 6.07 in PSS-co-MA coated Ti pins.

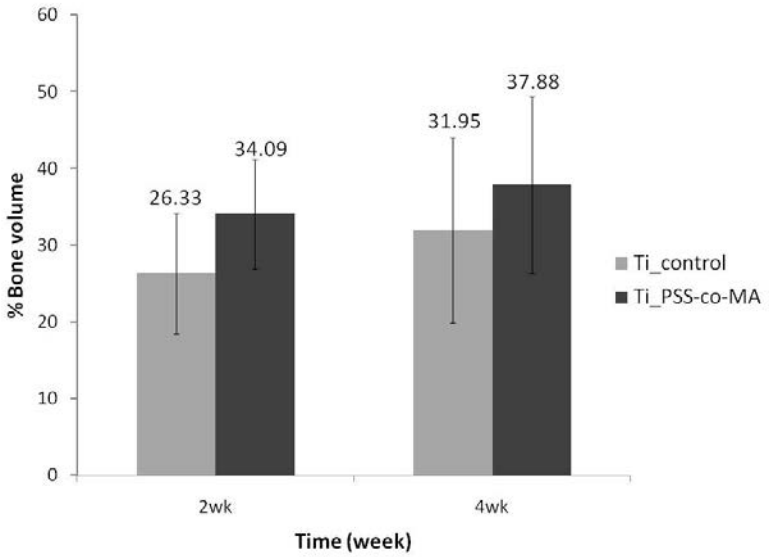

Figure 4.9 The percentages of bone volume around the titanium pins.

The histomorphometric analysis of new bone formation in bone volume term showed in the Figure 4.9. The amount of new bone formation at 0.1 mm. around the uncoated Ti pins and PSS-co-MA coated Ti pins were 26.33 ± 7.85 and 34.09 ± 7.13 at 2 week and 31.95 ± 12.06 and 37.88 ± 11.48 at 4 week, respectively. With the number available, no statistically significant differences between the groups were detected.

CHAPTER V
DISCUSSION AND CONCLUSION

DISCUSSION

Implant biocompatibility and ability to osseointegrate can be modified by certain factors such as surface composition, topography, degree of roughness, hydrophilicity and surface free energy [43]. In this study, the results indicated that modification of titanium surface with PSS-co-MA PEM film could improve the surface properties of titanium to support in vitro calcification and in vivo bone formation.

It has been shown that surface roughness and hydrophilicity of titanium surface could influence the differentiation and the expressions of an osteogenic phenotype of osteoblast-like cells [46, 47, 50, 53]. It is possible that rough surfaces provide larger the contact area between cells and materials interaction surface. However, the results from this study, as analyzed by SEM and AFM, did not show any significantly difference in the roughness between the PSS-co-MA coated and the uncoated surface. In addition, surface morphology of PSS-co-MA surface appeared to be smoother than the uncoated one, since the average roughness degree of PSS-co-MA coated surface was decreased from the uncoated surface. This phenomenon is possibly due to the effect of PEM films to fill the groove between the valleys of titanium surface. Therefore, roughness factor might not play roles in the osteoconductive of the PEM surface.

The surface wettability is another parameter that could affect the cellular behaviors at the cell-material interface. In this study, the contact angle is the used for evaluating the wettability of materials. From the results, the contact angle of uncoated Ti surface and PSS-co-MA coated Ti surface were 54.69 ± 0.86 degree and 50.82 ± 1.27 degree, respectively. The contact angle of both of uncoated Ti disc and PSS-co-MA coated Ti disc indicated the moderate hydrophilicity surface. However, PSS-co-MA PEM Ti surfaces possessed the higher hydrophilic property compared to the uncoated surfaces.

The higher hydrophilicity corresponded with the increased expressions of osteoblastic gene expression and the faster rate of *in vitro* calcification than lower hydrophilicity. These results imply that hydrophilicity might participate in promoting osseointegration. This study is in agreement with the results from many studies that suggested the role of hydrophilic surfaces to support thrombogenic properties [54], osteoblastic gene expression [55, 56], mineralization [53, 55] and bone-to-implant contact [48]. The wettability could also influence others cells-materials interactions

such as protein adsorption [15, 49, 57, 58]. However, there were reports showing the positive results of the hydrophobic surface compared to the hydrophilic one [45, 59]. Therefore, it is still unclear whether the positive results of PSS-co-MA are mainly due to the hydrophilic property.

When dental implant was placed into the bone, the surface was immediately contacted with blood. Platelets coagulation and fibrin network formation will occur on the implant surface and this network will function as a natural scaffold for the repair and/or regeneration process of bone. Moreover, the fibrin network generally contains several growth factors that can support wound healing around implants. It has been proposed that the thrombogenic property of titanium is important for a successful bone formation on dental implant [39, 40], therefore, the ability of titanium surface to support fibrin formation was investigated in this study. The fibrin formation was examined by dropping the whole blood on the surface. The results showed that higher amount of fibrin formations could be found on the PSS-co-MA coated Ti surface within the first 5 min compared to the control. The higher amount of fibrin network may indicate the better function of PEM surface for osseointegration. Milleret *et al.,* showed the well-structure and more density mesh of fibrin fiber on alkali treatment Ti surfaces after 10 min and 2 hour incubation [60]. Therefore, it is possible that the greater amount of fibrin on PSS-co-MA surface may result from the alkali properties of PSS-co-MA and alkali of distilled water in the PEM fabricated procedure.

MC3T3-E1 cells (osteoblast cell line) were used for study the interaction to PSS-co-MA surface. The expression of osteoblastic genes was used to represent biocompatibility of the implant since it has been shown that the expression of osteoblastic related genes has been shown to be correlated with stage of osteoblast differentiation [61]. The process of osteoblastic differentiation can be divided into 4 stages; proliferative, matrix formation, matrix maturation and calcification. There are many osteoblastic markers that were accepted for studying the biocompatibility of dental implants including Col-I, OPN, BSP and OC. Col-I is the early marker in bone formation, the high expression of Col-I occurs in the matrix formation stage. The effect of PSS-co-MA coated Ti surface on the induction of Col-I at day 5 indicated that PSS-co-MA surface promoted the differentiation rate towards the matrix formation stage. At day 10, the cells might already move into the matrix maturation stage, since cells seeded on PSS-co-MA surface stopped Col-I expression and increased the expressions of OPN, OC and BSP, markers of maturation stages of differentiation. Moreover, OC has been accepted as the late stage marker of osteoblastic differentiation [62]. Therefore, the increase expression of OC is generally implied that cell differentiated towards osteoblast. The osteoblast differentiation was supported by cells seeded on PSS-co-MA surface showed faster

rate of calcification compared to the uncoated Ti surface *in vitro*. This is in agreement with the previous study that found the PSS-co-MA coated on glass surface could promote the osteoblastic gene expression (OPN, BSP and OC) and developed faster rate of *in vitro* calcium deposition of MC3T3-E1 cells cultured on PSS-co-MA film compared the glass control [22].

The potential of PSS-co-MA PEM surface in osseointegration is also supported by the *in vivo* results. All of wound healed without wound infection or adverse reaction response indicating the biocompatibility of coated titanium pins. Since the PSS-co-MA seemed to be biocompatible to the tissue, no signs of bone resorption or foreign body reaction were observed.

For histological analysis, the titanium pins were cut cross sectionally. In general, the section of dental implant should be cut longitudinally. However, since the size of the pin is quite small, it is very difficult to obtain the proper longitudinal section. The disadvantage of cross section is that the whole area of titanium could not be seen at the same time. To compensate for the disadvantage, the serial section of the whole titanium pin was done. Approximately 20 sections could be obtained from each pin and the measurement was performed in all the sections.

In this study, titanium pins were placed into the medullary canal of the femur of the rat without the contact to compact bone. Therefore, the BIC value may have more advantage in determining the newly formed bone at the surface of the implant (contact osseointegration). The results indicated that percentages of BIC observed in PSS-co-MA coated Ti were higher significantly than the uncoated group at 2 weeks. However, no significant differences in BV were observed among the different groups. The results suggested that the PSS-co-MA PEM surface could accelerate the newly bone formed. However, due to the excellent property of titanium, the newly bone formation in the control showed no different compared to the coated surface at 4 weeks. Still, the quality of bone, such as the level of calcification, may still be different and this hypothesis is under investigated.

Furthermore, long term study of *in vivo* model as well as the experiment in larger size animal models is needed. Taken all the results together, it is possible that PSS-co-MA PEM films enhance the faster rate of bone formation compared to the uncoated Ti surface.

The factors that promoted bone formation of PSS-co-MA surface are still unknown. The evidences suggested that the surface charge of material surface may attract proteins and affect the gene expression. Many oral implant companies have launched products with surface charge such as Strauman (SLAtive® or Modified SLA;

Hydrophilic sandblast and acid-etched), Astratech (Osseospeed implant; Fluoridated implant) and Noble Biocare (Tiunite surface; anodized technique). So the surface charge on PSS-co-MA surface from polyelectrolyte coating may be the factor that stimulates up-regulations of genes expression, *in vitro* calcification and new bone formation *in vivo* study. Although the surface charge both of negatively and positively charged on material has been reported [38, 49], but the charged surface supports bone formation better than the uncharged surface.

From these results, all the evidence indicated the effectiveness of PSS-co-MA support bone formation on the other material such as the titanium surface. Angwarawong *et al.* [22] assumed that the effective PSS-co-MA might due to the effect of maleic acid. There were studies showing that maleic acid coating surface support to protein adsorption [63] especially fibronectin [64, 65]. Fibronectin is important to regulate cell growth, differentiation of osteoblast *in vitro* study [66] and early phase of osseointegration *in vivo* study [67]. Moreover, PSS-co-MA contain 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 hydroxyapatite and enamel has been reported [68-70]. However, the ability of PSS-co-MA film remains to elucidate.

CONCLUSION

In conclusion, the results from this study demonstrated that {(PDADMAC/PSS)$_4$/PDADMAC} PSS-co-MA PEM films coated on Ti disc could promote osteoblastic gene expressions and accelerate *in vitro* calcification of MC3T3-E1 cells. Moreover, the PSS-co-MA coated Ti pins enhanced the early new bone formations around the pins compared to the uncoated pins *in vivo* study. These results suggest the potentials of PEM technique for dental implant surface improvement to support the better osseointegration.

Acknowledgment

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APPENDICES

APPENDIX A

APPENDIX A

Figure A1 The technique determines the accuracy of the solution. All of solutions, PDADMAC, PSS and PSS-co-MA are clear. PDADMAC is positively charged and the others are negatively charged. When mix between PDADMAC and PSS or between PDADMAC and PSS-co-MA, solutions are opaque.

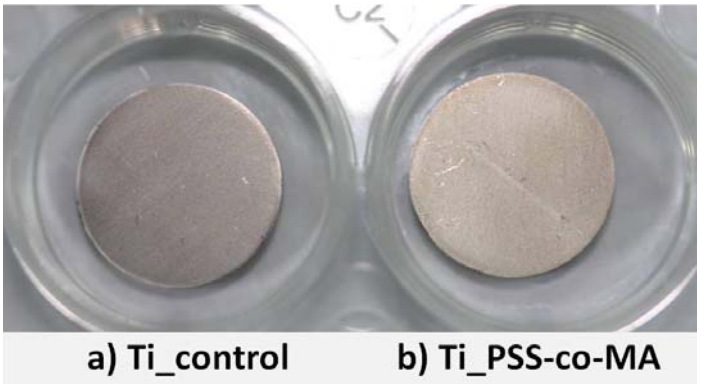

Figure A2 The color of titanium discs. a) Shows the color of uncoated Ti disc (control). b) Shows the color of PSS-co-MA coated Ti disc, it looks like in yellow-brown color.

Ti_control							
Element(%) / Sample no.	**1**	**2**	**3**	**4**	**5**	**mean**	**SD**
C	3.75	3.56	3.75	3.45	3.71	3.64	0.13
N	9.77	9.19	9.67	9.57	9.67	9.57	0.23
Si	0.4	0.41	0.42	0.32	0.36	0.38	0.04
Ti	86.07	86.84	86.16	86.66	86.27	86.40	0.33
Ti_PSS-co-MA							
Element(%) / Sample no.	**1**	**2**	**3**	**4**	**5**	**mean**	**SD**
C	4.77	4.69	4.73	5.29	5.2	4.94	0.29
N	10.33	10.14	10.4	10.65	10.45	10.39	0.19
Si	-	-	0.23	0.28	0.41	0.31	0.09
Ti	84.91	85.17	84.64	83.78	83.93	84.49	0.61

Table A1 Quantitative energy dispersive x-ray spectroscopy (EDS) analysis
The structures of polyelectrolyte are used in this study (show in Figure 3.1), consist of C and N elements. Therefore, the chemical compositions from EDS analysis was shown the PSS-co-MA coated Ti surfaces contained the percentage of C and N more than the uncoated Ti surfaces.

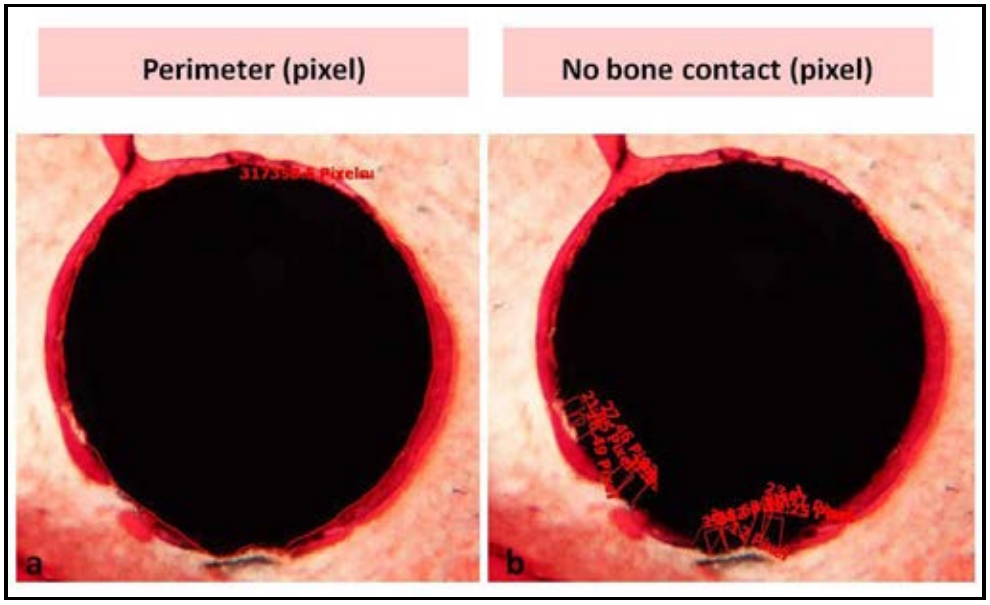

Figure A3 The figures show bone-to-implant contact (BIC) analysis. The sections are stained with alizarin red and take a photograph at magnification 10X. Percentage of BIC was calculated by AxioVision 4.8.1 software, Carl Zeiss, Microimaging GmbH, Germany. The percentage of BIC was calculated from the formular

"BIC (%) = Bone contact x 100 / perimeter"

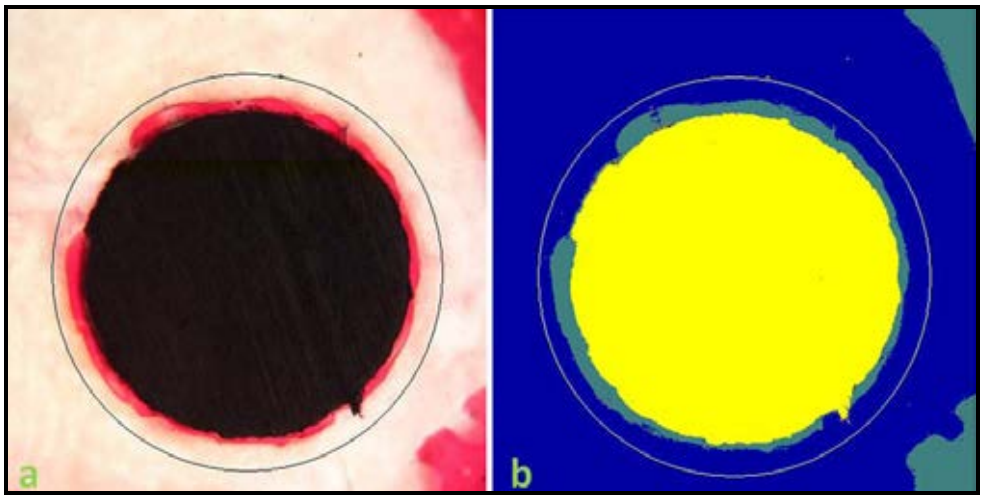

Figure A4 The figures show bone volume analysis. The sections are stained with alizarin red and take a photograph at magnification 10X. Percentage of BV was calculate by Image-Pro® Plus software, Version 6.0, Media Cybernetics, Inc., USA. The area of interesting (AOI) is 0.1 mm around the titanium surface (a). The titanium pin is yellow in color, the new bone is green in color and the background in the AOI is blue in color (b).

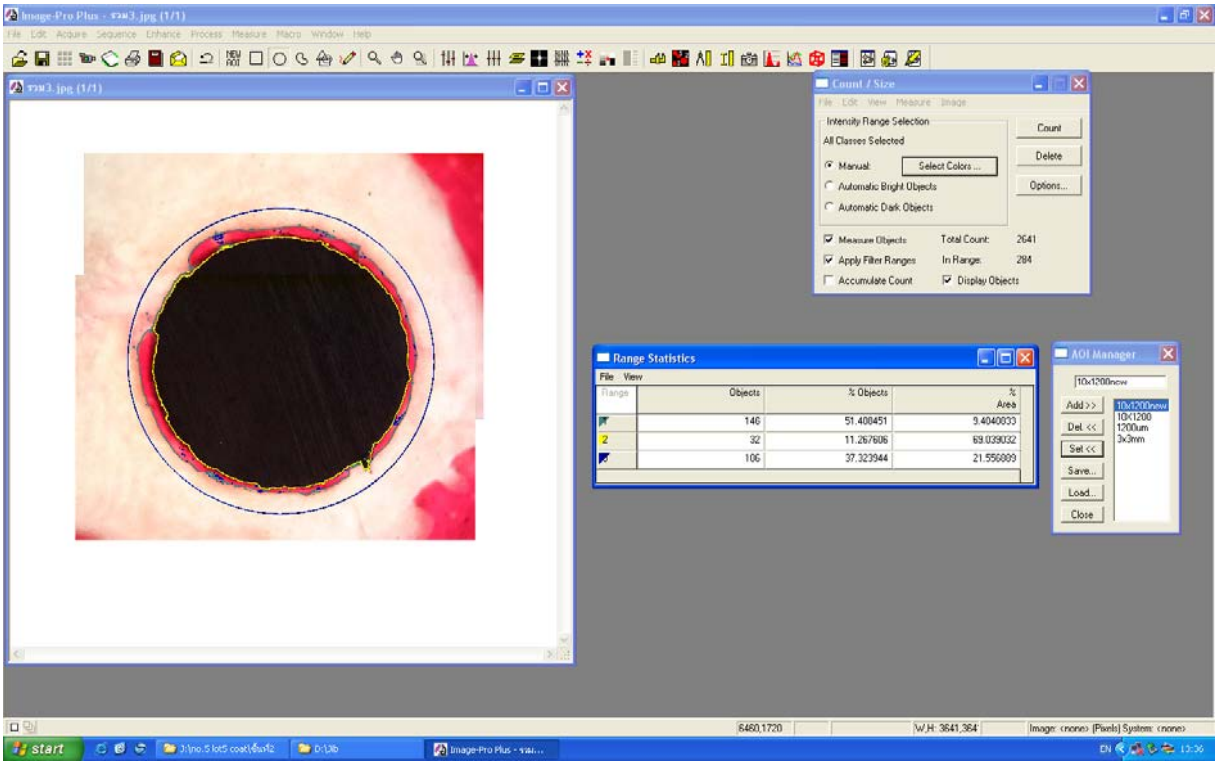

Figure A5 The figure shows the results of bone volume (BV) analysis from the Image-Pro® Plus software. The percentage of BV was calculated from the formular ***"BV (%) = percent of green area x 100 / (percent of green area + percent of blue area)"***

APPENDIX B

APPENDIX B

The statistic analysis of Average surface roughness (R_a) and the root mean square roughness (R_q)

Sample no.	Ti_control	
	Ra (nm)	Rq (nm)
1	145.23	180.1
2	142.64	183.28
3	158.89	201.75
Mean	**148.92**	**188.38**
S.D.	**8.73**	**11.69**

Sample no.	Ti_PSS-co-MA	
	Ra (nm)	Rq (nm)
1	138.82	183.16
2	137.09	174.14
3	152.18	189.75
Mean	**142.70**	**182.35**
S.D.	**8.26**	**7.84**

Average surface roughness (R_a)

NPar Tests

One-Sample Kolmogorov-Smirnov Test

group			roughness
Ti_control	N		3
	Normal Parameters[a,,b]	Mean	148.9200
		Std. Deviation	8.73085
	Most Extreme Differences	Absolute	.330
		Positive	.330
		Negative	-.236
	Kolmogorov-Smirnov Z		.572
	Asymp. Sig. (2-tailed)		.899
Ti_PSS-co-MA	N		3
	Normal Parameters[a,,b]	Mean	142.6967
		Std. Deviation	8.25823
	Most Extreme Differences	Absolute	.347
		Positive	.347
		Negative	-.249
	Kolmogorov-Smirnov Z		.602
	Asymp. Sig. (2-tailed)		.862

a. Test distribution is Normal.

b. Calculated from data.

T-Test

Group Statistics

	group	N	Mean	Std. Deviation	Std. Error Mean
roughness	Ti_control	3	148.9200	8.73085	5.04076
	Ti_PSS-co-MA	3	142.6967	8.25823	4.76789

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means	
		F	Sig.	t	df
roughness	Equal variances assumed	.017	.902	.897	4
	Equal variances not assumed			.897	3.988

Independent Samples Test

		t-test for Equality of Means		
		Sig. (2-tailed)	Mean Difference	Std. Error Difference
roughness	Equal variances assumed	.420	6.22333	6.93845
	Equal variances not assumed	.421	6.22333	6.93845

Independent Samples Test

		t-test for Equality of Means	
		95% Confidence Interval of the Difference	
		Lower	Upper
roughness	Equal variances assumed	-13.04088	25.48755
	Equal variances not assumed	-13.06439	25.51105

There are no significantly difference between uncoated Ti disc surfaces and PSS-co-MA coated Ti disc surfaces for *R*a (p>0.05)

The root mean square roughness (R_q)

NPar Tests

One-Sample Kolmogorov-Smirnov Test

group			Rq
Ti_control	N		3
	Normal Parameters[a,,b]	Mean	188.3767
		Std. Deviation	11.69028
	Most Extreme Differences	Absolute	.335
		Positive	.335
		Negative	-.239
	Kolmogorov-Smirnov Z		.581
	Asymp. Sig. (2-tailed)		.889
Ti_PSS-co-MA	N		3
	Normal Parameters[a,,b]	Mean	182.3500
		Std. Deviation	7.83646
	Most Extreme Differences	Absolute	.208
		Positive	.186
		Negative	-.208
	Kolmogorov-Smirnov Z		.360
	Asymp. Sig. (2-tailed)		.999

a. Test distribution is Normal.

b. Calculated from data.

T-Test

Group Statistics

	group	N	Mean	Std. Deviation	Std. Error Mean
Rq	Ti_control	3	188.3767	11.69028	6.74939
	Ti_PSS-co-MA	3	182.3500	7.83646	4.52438

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means	
		F	Sig.	t	df
Rq	Equal variances assumed	1.048	.364	.742	4
	Equal variances not assumed			.742	3.495

Independent Samples Test

		t-test for Equality of Means		
		Sig. (2-tailed)	Mean Difference	Std. Error Difference
Rq	Equal variances assumed	.499	6.02667	8.12553
	Equal variances not assumed	.505	6.02667	8.12553

Independent Samples Test

		t-test for Equality of Means	
		95% Confidence Interval of the Difference	
		Lower	Upper
Rq	Equal variances assumed	-16.53342	28.58676
	Equal variances not assumed	-17.87757	29.93090

There are no significantly difference between uncoated Ti disc surfaces and PSS-co-MA coated Ti disc surfaces for *Rq* (p>0.05)

The statistic analysis of contact angle

Sample no.	Contact angle	
	Ti_control	Ti_PSS-co-MA
1	55.3	51.9
2	53.4	48.0
3	55.3	52.2
4	55.3	51.9
5	54.6	51.3
6	54.2	50.5
7	55.3	49.5
8	53.1	50.8
9	54.9	51.2
10	55.5	50.9
mean	**54.69**	**50.82**
SD	**0.86**	**1.27**

\

NPar Tests

One-Sample Kolmogorov-Smirnov Test

group			contactangle
Ti_control	N		10
	Normal Parameters[a,,b]	Mean	54.6900
		Std. Deviation	.85823
	Most Extreme Differences	Absolute	.261
		Positive	.173
		Negative	-.261
	Kolmogorov-Smirnov Z		.827
	Asymp. Sig. (2-tailed)		.502
Ti_PSS-co-MA	N		10
	Normal Parameters[a,,b]	Mean	50.8200
		Std. Deviation	1.26561
	Most Extreme Differences	Absolute	.200
		Positive	.138
		Negative	-.200
	Kolmogorov-Smirnov Z		.633
	Asymp. Sig. (2-tailed)		.818

a. Test distribution is Normal.

One-Sample Kolmogorov-Smirnov Test

group			contactangle
Ti_control	N		10
	Normal Parameters[a,,b]	Mean	54.6900
		Std. Deviation	.85823
	Most Extreme Differences	Absolute	.261
		Positive	.173
		Negative	-.261
	Kolmogorov-Smirnov Z		.827
	Asymp. Sig. (2-tailed)		.502
Ti_PSS-co-MA	N		10
	Normal Parameters[a,,b]	Mean	50.8200
		Std. Deviation	1.26561
	Most Extreme Differences	Absolute	.200
		Positive	.138
		Negative	-.200
	Kolmogorov-Smirnov Z		.633
	Asymp. Sig. (2-tailed)		.818

a. Test distribution is Normal.

b. Calculated from data.

T-Test

Group Statistics

	group	N	Mean	Std. Deviation	Std. Error Mean
contactangle	Ti_control	10	54.6900	.85823	.27140
	Ti_PSS-co-MA	10	50.8200	1.26561	.40022

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means	
		F	Sig.	t	df
contactangle	Equal variances assumed	.455	.508	8.003	18
	Equal variances not assumed			8.003	15.832

Independent Samples Test

		t-test for Equality of Means		
		Sig. (2-tailed)	Mean Difference	Std. Error Difference
contactangle	Equal variances assumed	.000	3.87000	.48356
	Equal variances not assumed	.000	3.87000	.48356

Independent Samples Test

		t-test for Equality of Means	
		95% Confidence Interval of the Difference	
		Lower	Upper
contactangle	Equal variances assumed	2.85407	4.88593
	Equal variances not assumed	2.84401	4.89599

Results showed that PSS-co-MA coated Ti surface has significant lower contact angle than the uncoated Ti disc. ($p < 0.01$).

The statistic analysis of gene expressions

Col-1 expressions at day 5

No.	Relative expression of Col-1 at day 5	
	Ti_control	Ti_PSS-co-MA
1	1.000000000	3.563488900
2	1.000000000	6.080714300

NPar Tests

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
expression	control	2	1.50	3.00
	coat	2	3.50	7.00
	Total	4		

Test Statistics[b]

	expression
Mann-Whitney U	.000
Wilcoxon W	3.000
Z	-1.633
Asymp. Sig. (2-tailed)	.102
Exact Sig. [2*(1-tailed Sig.)]	.333[a]

a. Not corrected for ties.

b. Grouping Variable: group

Result showed the expressions of Col-1 were significant increased in cell cultured on PSS-co-MA coated Ti surfaces when compared to uncoated Ti discs at day 5.

Col-1 expressions at day 10

No.	Relative expression of Col-1 at day 10	
	Ti_control	Ti_PSS-co-MA
1	1.000000000	0.014179987
2	1.000000000	0.010821168

NPar Tests

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
expression	control	2	3.50	7.00
	coat	2	1.50	3.00
	Total	4		

Test Statistics[b]

	expression
Mann-Whitney U	.000
Wilcoxon W	3.000
Z	-1.633
Asymp. Sig. (2-tailed)	.102
Exact Sig. [2*(1-tailed Sig.)]	.333[a]

a. Not corrected for ties.

b. Grouping Variable: group

Result showed the expressions of Col-1 in cell cultured on PSS-co-MA coated Ti surfaces were significant lesser than the uncoated Ti discs at day 10.

OPN expressions at day 5

No.	Relative expression of OPN at day 5	
	Ti_control	Ti_PSS-co-MA
1	1.000000000	1.071773463
2	1.000000000	0.858565436

NPar Tests

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
expression	control	2	2.50	5.00
	coat	2	2.50	5.00
	Total	4		

Test Statistics[b]

	expression
Mann-Whitney U	2.000
Wilcoxon W	5.000
Z	.000
Asymp. Sig. (2-tailed)	1.000
Exact Sig. [2*(1-tailed Sig.)]	1.000[a]

a. Not corrected for ties.

b. Grouping Variable: group

Result showed the expressions of OPN in cell cultured on uncoated Ti surfaces were not significantly when compare with PSS-co-MA coated Ti surfaces at day 5.

OPN expressions at day 10

No.	Relative expression of OPN at day 10	
	Ti_control	Ti_PSS-co-MA
1	1.000000000	2.056227653
2	1.000000000	2.602683711

NPar Tests

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
expression	control	2	1.50	3.00
	coat	2	3.50	7.00
	Total	4		

Test Statistics[b]

	expression
Mann-Whitney U	.000
Wilcoxon W	3.000
Z	-1.633
Asymp. Sig. (2-tailed)	.102
Exact Sig. [2*(1-tailed Sig.)]	.333[a]

a. Not corrected for ties.

b. Grouping Variable: group

Result showed the expressions of OPN were significant increased in cell cultured on PSS-co-MA coated Ti surfaces when compared to uncoated Ti discs at day 10.

BSP expressions at day 5

No.	Relative expression of BSP at day 5	
	Ti_control	Ti_PSS-co-MA
1	1.000000000	2.549121255
2	1.000000000	1.591072968

NPar Tests

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
expression	control	2	1.50	3.00
	coat	2	3.50	7.00
	Total	4		

Test Statistics[b]

	expression
Mann-Whitney U	.000
Wilcoxon W	3.000
Z	-1.633
Asymp. Sig. (2-tailed)	.102
Exact Sig. [2*(1-tailed Sig.)]	.333[a]

a. Not corrected for ties.

b. Grouping Variable: group

Result showed the expressions of BSP were significant increased in cell cultured on PSS-co-MA coated Ti surfaces when compared to uncoated Ti discs at day 5.

BSP expressions at day 10

No.	Relative expression of BSP at day 10	
	Ti_control	Ti_PSS-co-MA
1	1.000000000	1.334392700
2	1.000000000	7.113634800

NPar Tests

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
expression	control	2	1.50	3.00
	coat	2	3.50	7.00
	Total	4		

Test Statistics[b]

	expression
Mann-Whitney U	.000
Wilcoxon W	3.000
Z	-1.633
Asymp. Sig. (2-tailed)	.102
Exact Sig. [2*(1-tailed Sig.)]	.333[a]

a. Not corrected for ties.

b. Grouping Variable: group

Result showed the expressions of BSP were significant increased in cell cultured on PSS-co-MA coated Ti surfaces when compared to uncoated Ti discs at day 10.

OC expressions at day 5

No.	Relative expression of OC at day5	
	Ti_control	Ti_PSS-co-MA
1	1.000000000	0.482968164
2	1.000000000	1.591072968

NPar Tests

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
expression	control	2	2.50	5.00
	coat	2	2.50	5.00
	Total	4		

Test Statistics[b]

	expression
Mann-Whitney U	2.000
Wilcoxon W	5.000
Z	.000
Asymp. Sig. (2-tailed)	1.000
Exact Sig. [2*(1-tailed Sig.)]	1.000[a]

a. Not corrected for ties.

b. Grouping Variable: group

Result showed the expressions of OC in cell cultured on uncoated Ti surfaces were not significantly when compare with PSS-co-MA coated Ti surfaces at day 5.

OC expressions at day 10

No.	Relative expression of OC at day 10	
	Ti_control	Ti_PSS-co-MA
1	1.000000000	5.230895547
2	1.000000000	8.476174900

NPar Tests

Mann-Whitney Test

Ranks

	group	N	Mean Rank	Sum of Ranks
expression	control	2	1.50	3.00
	coat	2	3.50	7.00
	Total	4		

Test Statistics[b]

	expression
Mann-Whitney U	.000
Wilcoxon W	3.000
Z	-1.633
Asymp. Sig. (2-tailed)	.102
Exact Sig. [2*(1-tailed Sig.)]	.333[a]

a. Not corrected for ties.

b. Grouping Variable: group

Result showed the expressions of OC were significant increased in cell cultured on PSS-co-MA coated Ti surfaces when compared to uncoated Ti discs at day 10.

The statistic analysis of Alizarin red-S staining at day 15

No.	Relative absorbance at 570 nm.	
	Ti_control	Ti_PSS-co-MA
1	1.0000000	1.6865672
2	1.0000000	1.8953722
3	1.0000000	2.0531561

NPar Tests

One-Sample Kolmogorov-Smirnov Test

group			Alizarinred
control	N		3
	Normal Parameters[a,,b]	Mean	1.000000000
		Std. Deviation	.0000000000[c]
coat	N		3
	Normal Parameters[a,,b]	Mean	1.878365181
		Std. Deviation	.1838852911
	Most Extreme Differences	Absolute	.204
		Positive	.185
		Negative	-.204
	Kolmogorov-Smirnov Z		.352
	Asymp. Sig. (2-tailed)		1.000

a. Test distribution is Normal.

b. Calculated from data.

c. The distribution has no variance for this variable. One-Sample Kolmogorov-Smirnov Test cannot be performed.

T-Test

Group Statistics

	group	N	Mean	Std. Deviation	Std. Error Mean
Alizarinred	control	3	1.000000000	.0000000000	.0000000000
	coat	3	1.878365181	.1838852911	.1061662223

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means	
		F	Sig.	t	df
Alizarinred	Equal variances assumed	5.280	.083	-8.273	4
	Equal variances not assumed			-8.273	2.000

Independent Samples Test

		t-test for Equality of Means		
		Sig. (2-tailed)	Mean Difference	Std. Error Difference
Alizarinred	Equal variances assumed	.001	-.8783651810	.1061662223
	Equal variances not assumed	.014	-.8783651810	.1061662223

Independent Samples Test

		t-test for Equality of Means	
		95% Confidence Interval of the Difference	
		Lower	Upper
Alizarinred	Equal variances assumed	-1.1731298E0	-.5836004927
	Equal variances not assumed	-1.3351615E0	-.4215687947

The results showed the relative absorbance of PSS-co-MA coated Ti surface was significant greater than uncoated Ti discs (p<0.05).

The statistic analysis of examiner calibration

BIC	ก่อน	หลัง
BIC1	78.47	84.31
BIC2	86.49	86.26
BIC3	58.17	57.62
BIC4	41.2	37.23
BIC5	95.6	95.41
BIC6	53.19	46.61
BIC7	94.51	92.33
BIC8	54.43	60.64
BIC9	90.78	87.25
BIC10	95.02	92.93
BIC11	85.5	84.87
BIC12	84.79	83.95
BIC13	74.71	70.83
BIC14	80.13	78.7
BIC15	95.45	96.24
BIC16	92.33	92.14
BIC17	81.54	82.33
BIC18	72.37	73.2
BIC19	82.56	83.87
BIC20	85.19	88.12
BIC21	90.7	93.12
BIC22	86.57	84.93
BIC23	40.32	40.53
BIC24	43.65	44.29
BIC25	61.67	57.43
BIC26	44.91	43.55
BIC27	59.12	62.03
BIC28	85.54	84.87
BIC29	42.78	40.14
BIC30	67.76	64.9
BIC31	53.69	58.84
BIC32	49.69	56

BV	ก่อน	หลัง
BV1	65.22	63.3
BV2	56.26	57.25
BV3	45.81	43.69
BV4	49.54	51.45
BV5	73.25	70.91
BV6	40.16	39.27
BV7	35.43	39.59
BV8	66.86	62.17
BV9	34.2	31.27
BV10	40.16	44.66
BV11	32.79	34.2
BV12	62.43	62.55
BV13	78.09	83.98
BV14	52.33	46.63
BV15	52.96	52.33
BV16	78.03	73.67
BV17	70.82	69.56
BV18	82.33	87.62
BV19	26.42	31.64
BV20	48.17	50.03
BV21	47.77	47.98
BV22	54.84	53.72
BV23	44.34	46.77
BV24	64.01	70.12
BV25	42.8	39.98
BV26	61.12	56.36
BV27	77.7	76.79
BV28	58.39	56.49
BV29	70.12	71.88
BV30	50.06	53.94
BV31	45.81	49.54
BV32	65.34	63.01

The statistic analysis of BIC calibration was compared between before and after in one examiner.

NPar Tests

One-Sample Kolmogorov-Smirnov Test

		before	after
N		32	32
Normal Parameters[a,,b]	Mean	72.1509	72.0459
	Std. Deviation	18.85669	18.99562
Most Extreme Differences	Absolute	.164	.206
	Positive	.108	.101
	Negative	-.164	-.206
Kolmogorov-Smirnov Z		.927	1.165
Asymp. Sig. (2-tailed)		.356	.133

a. Test distribution is Normal.

b. Calculated from data.

T-Test

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	before	72.1509	32	18.85669	3.33342
	after	72.0459	32	18.99562	3.35798

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	before & after	32	.986	.000

Paired Samples Test

		Paired Differences				
					95% Confidence Interval of the Difference	
		Mean	Std. Deviation	Std. Error Mean	Lower	Upper
Pair 1	before - after	.10500	3.12665	.55272	-1.02228	1.23228

Paired Samples Test

		t	df	Sig. (2-tailed)
Pair 1	before - after	.190	31	.851

Correlations

Correlations

		before	after
before	Pearson Correlation	1	.986**
	Sig. (2-tailed)		.000
	N	32	32
after	Pearson Correlation	.986**	1
	Sig. (2-tailed)	.000	
	N	32	32

**. Correlation is significant at the 0.01 level (2-tailed).

The statistic analysis of BV calibration was compared between before and after in one examiner.

NPar Tests

One-Sample Kolmogorov-Smirnov Test

		before	after
N		32	32
Normal Parameters[a,,b]	Mean	55.4237	55.6984
	Std. Deviation	14.84643	14.69231
Most Extreme Differences	Absolute	.079	.083
	Positive	.079	.083
	Negative	-.062	-.077
Kolmogorov-Smirnov Z		.444	.469
Asymp. Sig. (2-tailed)		.989	.980

a. Test distribution is Normal.

b. Calculated from data.

T-Test

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	before	55.4237	32	14.84643	2.62450
	after	55.6984	32	14.69231	2.59726

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	before & after	32	.974	.000

Paired Samples Test

		Paired Differences				
					95% Confidence Interval of the Difference	
		Mean	Std. Deviation	Std. Error Mean	Lower	Upper
Pair 1	before - after	-.27469	3.36369	.59462	-1.48743	.93805

Paired Samples Test

		t	df	Sig. (2-tailed)
Pair 1	before - after	-.462	31	.647

Correlations

Correlations

		before	after
before	Pearson Correlation	1	.974**
	Sig. (2-tailed)		.000
	N	32	32
after	Pearson Correlation	.974**	1
	Sig. (2-tailed)	.000	
	N	32	32

**. Correlation is significant at the 0.01 level (2-tailed).

The statistic analysis of Bone-to-implant contact (BIC) and Bone volume (BV)

Rat no.	% BIC at 2 wk	
	Ti_control	Ti_PSS-co-MA
1	60.62	71.01
2	42.40	76.14
3	48.24	57.71
4	50.82	60.26
Rat no.	% BIC at 4 wk	
	Ti_control	Ti_PSS-co-MA
5	70.08	83.60
6	78.70	78.06
7	49.37	81.74
8	71.58	81.74

Rat no.	BV at 2 wk	
	Ti_control	Ti_PSS-co-MA
1	35.84	44.74
2	16.92	31.06
3	27.97	30.88
4	24.59	29.67
Rat no.	BV at 4 wk	
	Ti_control	Ti_PSS-co-MA
5	46.78	51.51
6	32.65	28.69
7	17.29	43.24
8	31.09	28.07

The statistic analysis of BIC was compared between the uncoated and the coated surface at 2 week.

NPar Tests

One-Sample Kolmogorov-Smirnov Test

group			BIC
control	N		4
	Normal Parameters[a,,b]	Mean	50.5200
		Std. Deviation	7.59896
	Most Extreme Differences	Absolute	.234
		Positive	.234
		Negative	-.158
	Kolmogorov-Smirnov Z		.469
	Asymp. Sig. (2-tailed)		.981
coat	N		4
	Normal Parameters[a,,b]	Mean	66.2800
		Std. Deviation	8.74219
	Most Extreme Differences	Absolute	.254
		Positive	.254
		Negative	-.206
	Kolmogorov-Smirnov Z		.509
	Asymp. Sig. (2-tailed)		.958

a. Test distribution is Normal.

b. Calculated from data.

T-Test

Group Statistics

	group	N	Mean	Std. Deviation	Std. Error Mean
BIC	control	4	50.5200	7.59896	3.79948
	coat	4	66.2800	8.74219	4.37110

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means	
		F	Sig.	t	df
BIC	Equal variances assumed	.646	.452	-2.721	6
	Equal variances not assumed			-2.721	5.886

Independent Samples Test

		t-test for Equality of Means		
		Sig. (2-tailed)	Mean Difference	Std. Error Difference
BIC	Equal variances assumed	.035	-15.76000	5.79159
	Equal variances not assumed	.035	-15.76000	5.79159

Independent Samples Test

		t-test for Equality of Means	
		95% Confidence Interval of the Difference	
		Lower	Upper
BIC	Equal variances assumed	-29.93152	-1.58848
	Equal variances not assumed	-29.99838	-1.52162

The results showed BIC of PSS-co-MA coated Ti pins was significant greater than the uncoated pins at 2 weeks ($p < 0.05$).

The statistic analysis of BIC was compared between the uncoated and the coated surface at 4 week.

NPar Tests

One-Sample Kolmogorov-Smirnov Test

group			BIC
control	N		4
	Normal Parameters[a.,b]	Mean	67.4325
		Std. Deviation	12.61509
	Most Extreme Differences	Absolute	.333
		Positive	.186
		Negative	-.333
	Kolmogorov-Smirnov Z		.666
	Asymp. Sig. (2-tailed)		.766
coat	N		4
	Normal Parameters[a.,b]	Mean	78.3275
		Std. Deviation	6.06548
	Most Extreme Differences	Absolute	.232
		Positive	.192
		Negative	-.232
	Kolmogorov-Smirnov Z		.465
	Asymp. Sig. (2-tailed)		.982

a. Test distribution is Normal.

b. Calculated from data.

T-Test

Group Statistics

	group	N	Mean	Std. Deviation	Std. Error Mean
BIC	control	4	67.4325	12.61509	6.30754
	coat	4	78.3275	6.06548	3.03274

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means	
		F	Sig.	t	df
BIC	Equal variances assumed	1.418	.279	-1.557	6
	Equal variances not assumed			-1.557	4.317

Independent Samples Test

		t-test for Equality of Means		
		Sig. (2-tailed)	Mean Difference	Std. Error Difference
BIC	Equal variances assumed	.171	-10.89500	6.99876
	Equal variances not assumed	.189	-10.89500	6.99876

Independent Samples Test

		t-test for Equality of Means	
		95% Confidence Interval of the Difference	
		Lower	Upper
BIC	Equal variances assumed	-28.02035	6.23035
	Equal variances not assumed	-29.77713	7.98713

The results showed BIC of PSS-co-MA coated Ti pins was greater than the uncoated pins but not significant at 4 weeks ($p > 0.05$).

The statistic analysis of BIC was compared between 2 week and 4 week in the uncoated group.

NPar Tests

One-Sample Kolmogorov-Smirnov Test

time			BIC
2 wk	N		4
	Normal Parameters[a,,b]	Mean	50.5200
		Std. Deviation	7.59896
	Most Extreme Differences	Absolute	.234
		Positive	.234
		Negative	-.158
	Kolmogorov-Smirnov Z		.469
	Asymp. Sig. (2-tailed)		.981
4 wk	N		4
	Normal Parameters[a,,b]	Mean	67.4325
		Std. Deviation	12.61509
	Most Extreme Differences	Absolute	.333
		Positive	.186
		Negative	-.333
	Kolmogorov-Smirnov Z		.666
	Asymp. Sig. (2-tailed)		.766

a. Test distribution is Normal.

b. Calculated from data.

T-Test

Group Statistics

	time	N	Mean	Std. Deviation	Std. Error Mean
BIC	2 wk	4	50.5200	7.59896	3.79948
	4 wk	4	67.4325	12.61509	6.30754

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means	
		F	Sig.	t	df
BIC	Equal variances assumed	.815	.402	-2.297	6
	Equal variances not assumed			-2.297	4.924

Independent Samples Test

		t-test for Equality of Means		
		Sig. (2-tailed)	Mean Difference	Std. Error Difference
BIC	Equal variances assumed	.061	-16.91250	7.36350
	Equal variances not assumed	.071	-16.91250	7.36350

Independent Samples Test

		t-test for Equality of Means	
		95% Confidence Interval of the Difference	
		Lower	Upper
BIC	Equal variances assumed	-34.93034	1.10534
	Equal variances not assumed	-35.92942	2.10442

The results showed BIC in uncoated group at 4 week was greater at 2 week, but not significant ($p > 0.05$).

The statistic analysis of BIC was compared between 2 week and 4 week in the coated group.

NPar Tests

One-Sample Kolmogorov-Smirnov Test

time			BIC
2 wk	N		4
	Normal Parameters[a,,b]	Mean	66.2800
		Std. Deviation	8.74219
	Most Extreme Differences	Absolute	.254
		Positive	.254
		Negative	-.206
	Kolmogorov-Smirnov Z		.509
	Asymp. Sig. (2-tailed)		.958
4 wk	N		4
	Normal Parameters[a,,b]	Mean	78.3275
		Std. Deviation	6.06548
	Most Extreme Differences	Absolute	.232
		Positive	.192
		Negative	-.232
	Kolmogorov-Smirnov Z		.465
	Asymp. Sig. (2-tailed)		.982

a. Test distribution is Normal.

b. Calculated from data.

T-Test

Group Statistics

	time	N	Mean	Std. Deviation	Std. Error Mean
BIC	2 wk	4	66.2800	8.74219	4.37110
	4 wk	4	78.3275	6.06548	3.03274

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means	
		F	Sig.	t	df
BIC	Equal variances assumed	2.037	.203	-2.265	6
	Equal variances not assumed			-2.265	5.345

Independent Samples Test

		t-test for Equality of Means		
		Sig. (2-tailed)	Mean Difference	Std. Error Difference
BIC	Equal variances assumed	.064	-12.04750	5.32015
	Equal variances not assumed	.070	-12.04750	5.32015

Independent Samples Test

		t-test for Equality of Means	
		95% Confidence Interval of the Difference	
		Lower	Upper
BIC	Equal variances assumed	-25.06544	.97044
	Equal variances not assumed	-25.46216	1.36716

The results showed BIC in coated group at 4 week was greater at 2 week, but not significant ($p > 0.05$).

The statistic analysis of BV was compared between the uncoated and the coated surface at 2 week.

NPar Tests

One-Sample Kolmogorov-Smirnov Test

group			BV
control	N		4
	Normal Parameters[a,,b]	Mean	26.3300
		Std. Deviation	7.84656
	Most Extreme Differences	Absolute	.167
		Positive	.167
		Negative	-.162
	Kolmogorov-Smirnov Z		.334
	Asymp. Sig. (2-tailed)		1.000
coat	N		4
	Normal Parameters[a,,b]	Mean	34.0875
		Std. Deviation	7.12844
	Most Extreme Differences	Absolute	.414
		Positive	.414
		Negative	-.268
	Kolmogorov-Smirnov Z		.829
	Asymp. Sig. (2-tailed)		.498

a. Test distribution is Normal.

b. Calculated from data.

T-Test

Group Statistics

	group	N	Mean	Std. Deviation	Std. Error Mean
BV	control	4	26.3300	7.84656	3.92328
	coat	4	34.0875	7.12844	3.56422

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means	
		F	Sig.	t	df
BV	Equal variances assumed	.007	.934	-1.464	6
	Equal variances not assumed			-1.464	5.946

Independent Samples Test

		t-test for Equality of Means		
		Sig. (2-tailed)	Mean Difference	Std. Error Difference
BV	Equal variances assumed	.194	-7.75750	5.30054
	Equal variances not assumed	.194	-7.75750	5.30054

Independent Samples Test

		t-test for Equality of Means	
		95% Confidence Interval of the Difference	
		Lower	Upper
BV	Equal variances assumed	-20.72747	5.21247
	Equal variances not assumed	-20.75631	5.24131

The results showed BV of PSS-co-MA coated Ti pins was greater than the uncoated pins but not significant at 2 weeks ($p > 0.05$).

The statistic analysis of BV was compared between the uncoated and the coated surface at 4 week.

NPar Tests

One-Sample Kolmogorov-Smirnov Test

group			BV
control	N		4
	Normal Parameters[a,,b]	Mean	31.9525
		Std. Deviation	12.05645
	Most Extreme Differences	Absolute	.227
		Positive	.227
		Negative	-.221
	Kolmogorov-Smirnov Z		.454
	Asymp. Sig. (2-tailed)		.986
coat	N		4
	Normal Parameters[a,,b]	Mean	37.8775
		Std. Deviation	11.47750
	Most Extreme Differences	Absolute	.288
		Positive	.288
		Negative	-.196
	Kolmogorov-Smirnov Z		.577
	Asymp. Sig. (2-tailed)		.894

a. Test distribution is Normal.

b. Calculated from data.

T-Test

Group Statistics

	group	N	Mean	Std. Deviation	Std. Error Mean
BV	control	4	31.9525	12.05645	6.02823
	coat	4	37.8775	11.47750	5.73875

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means	
		F	Sig.	t	df
BV	Equal variances assumed	.157	.705	-.712	6
	Equal variances not assumed			-.712	5.986

Independent Samples Test

		t-test for Equality of Means		
		Sig. (2-tailed)	Mean Difference	Std. Error Difference
BV	Equal variances assumed	.503	-5.92500	8.32302
	Equal variances not assumed	.503	-5.92500	8.32302

Independent Samples Test

		t-test for Equality of Means	
		95% Confidence Interval of the Difference	
		Lower	Upper
BV	Equal variances assumed	-26.29071	14.44071
	Equal variances not assumed	-26.30265	14.45265

The results showed BV of PSS-co-MA coated Ti pins was greater than the uncoated pins but not significant at 4 weeks ($p > 0.05$).

The statistic analysis of BV was compared between 2 week and 4 week in the uncoated group.

NPar Tests

One-Sample Kolmogorov-Smirnov Test

time			BV
2 wk	N		4
	Normal Parameters[a,,b]	Mean	26.3300
		Std. Deviation	7.84656
	Most Extreme Differences	Absolute	.167
		Positive	.167
		Negative	-.162
	Kolmogorov-Smirnov Z		.334
	Asymp. Sig. (2-tailed)		1.000
4 wk	N		4
	Normal Parameters[a,,b]	Mean	31.9525
		Std. Deviation	12.05645
	Most Extreme Differences	Absolute	.227
		Positive	.227
		Negative	-.221
	Kolmogorov-Smirnov Z		.454
	Asymp. Sig. (2-tailed)		.986

a. Test distribution is Normal.

b. Calculated from data.

T-Test

Group Statistics

	time	N	Mean	Std. Deviation	Std. Error Mean
BV	2 wk	4	26.3300	7.84656	3.92328
	4 wk	4	31.9525	12.05645	6.02823

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means	
		F	Sig.	t	df
BV	Equal variances assumed	.225	.652	-.782	6
	Equal variances not assumed			-.782	5.155

Independent Samples Test

		t-test for Equality of Means		
		Sig. (2-tailed)	Mean Difference	Std. Error Difference
BV	Equal variances assumed	.464	-5.62250	7.19247
	Equal variances not assumed	.469	-5.62250	7.19247

Independent Samples Test

		t-test for Equality of Means	
		95% Confidence Interval of the Difference	
		Lower	Upper
BV	Equal variances assumed	-23.22184	11.97684
	Equal variances not assumed	-23.94557	12.70057

The results showed BV in uncoated group at 4 week was greater at 2 week, but not significant ($p > 0.05$).

The statistic analysis of BV was compared between 2 week and 4 week in the coated group.

NPar Tests

One-Sample Kolmogorov-Smirnov Test

time			BV
2 wk	N		4
	Normal Parameters[a,,b]	Mean	34.0875
		Std. Deviation	7.12844
	Most Extreme Differences	Absolute	.414
		Positive	.414
		Negative	-.268
	Kolmogorov-Smirnov Z		.829
	Asymp. Sig. (2-tailed)		.498
4 wk	N		4
	Normal Parameters[a,,b]	Mean	37.8775
		Std. Deviation	11.47750
	Most Extreme Differences	Absolute	.288
		Positive	.288
		Negative	-.196
	Kolmogorov-Smirnov Z		.577
	Asymp. Sig. (2-tailed)		.894

a. Test distribution is Normal.

b. Calculated from data.

T-Test

Group Statistics

	time	N	Mean	Std. Deviation	Std. Error Mean
BV	2 wk	4	34.0875	7.12844	3.56422
	4 wk	4	37.8775	11.47750	5.73875

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means	
		F	Sig.	t	df
BV	Equal variances assumed	2.846	.143	-.561	6
	Equal variances not assumed			-.561	5.015

Independent Samples Test

		t-test for Equality of Means		
		Sig. (2-tailed)	Mean Difference	Std. Error Difference
BV	Equal variances assumed	.595	-3.79000	6.75551
	Equal variances not assumed	.599	-3.79000	6.75551

Independent Samples Test

		t-test for Equality of Means	
		95% Confidence Interval of the Difference	
		Lower	Upper
BV	Equal variances assumed	-20.32013	12.74013
	Equal variances not assumed	-21.14032	13.56032

The results showed BV in coated group at 4 week was greater at 2 week, but not significant ($p > 0.05$).

VITA

Miss Watchawadee Hoonwichit was born in Nakhon Nayok, Thailand on November 22, 1982. In 2006, she was conferred the degree of Doctor of Dental Surgery (D.S.S) which second class honor from Faculty of Dentistry, Prince of Songkla University. After graduation, she worked as a dentist at Nakae Hospital, Nakhon Phanom, Thailand (2006-2007) and Nakhonnayok Provincial Public Health office, Nakhon Nayok, Thailand (2008).

In 2009, she started her post-graduated study for the Master of Science in Prosthodontics Program at the Faculty of Dentistry, Chulalongkorn University.