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OPN IMMOBIZATION ON SURFACE-GRAFTED POLY(ACRYLIC ACID)BRUSHES TO PROMOTE OSTEOBLAST ADHESION AND PROLIFERATION

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A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	OPN IMMOBIZATION ON SURFACE-GRAFTEDPOLY(ACRYLIC ACID)BRUSHES TO PROMOTE OSTEOBLAST ADHESION AND PROLIFERATION
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พณิฎฐา ดำส่งแสง : การตรึงโอพีเอ็นบนพอลิอะคริลิกแอซิดบรัชที่กราฟต์บนผิวเพื่อส่งเสริมการยึดติดและการเจริญของเซลล์สร้างกระดูก (OPN IMMOBIZATION ON SURFACE-GRAFTEDPOLY(ACRYLIC ACID)BRUSHES TO PROMOTE OSTEOBLAST ADHESION AND PROLIFERATION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.วรวีร์ โฮเว่น, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ทพ. ดร.ประสิทธิ์ ภวสันต์, 43 หน้า.

ในงานวิจัยนี้ได้กราฟต์พอลิอะคริลิกแอซิด (พีเอเอ) บรัช บนผิวกระจกโดยอาศัยปฏิกิริยาพอลิเมอไรเซชันริเริ่มจากพื้นผิวผ่านกลไกแบบ Reversible Addition-Fragmentation Chain Transfer (RAFT) ผลการวิเคราะห์ PAA ที่เกิดขึ้นในสารละลายด้วยเทคนิค ^{1}H-NMR แสดงให้เห็นว่าสามารถควบคุมการเกิดปฏิกิริยาพอลิเมอไรเซชันได้ดี จากนั้นตรึงโปรตีนออสติโอพอนติน (โอพีเอ็น) ที่สังเคราะห์จากต้นยาสูบลงบนพื้นผิวที่กราฟต์ด้วยพีเอเอบรัช โดยใช้ EDC/NHS เป็นรีเอเจนต์คู่ควบ สามารถยืนยันการดัดแปรพื้นผิวกระจกแต่ละขั้นตอนได้โดยการวัดมุมสัมผัสของน้ำและเทคนิค FT-IR การวิเคราะห์ด้วยเทคนิค FT-IR, XPS และ AFM จากการหาปริมาณโอพีเอ็นที่ตรึงบนพื้นผิวด้วยเทคนิค ELISA assay พบว่าพื้นผิวดังกล่าวสามารถตรึงโอพีเอ็นลงบนผิวได้มากกว่า 95 เปอร์เซ็นต์ จากการวิเคราะห์ด้วยปฏิกิริยาลูกโซ่พอลิเมอเรส (qPCR) ชี้ให้เห็นถึงการแสดงออกของยีนต่างๆ ได้แก่ Collagen I (Col I), Osterix (Osx) และ Runt-related transcription factor 2 (Runx2) พบว่าพื้นผิวที่ตรึงโปรตีน OPN สามารถส่งเสริมการแปรสภาพไปเป็นเซลล์กระดูกได้ดีกว่าพื้นผิวกระจกเปล่าและพื้นผิวที่ตรึงด้วยโปรตีนเจลาติน นอกจากนี้สามารถวัดปริมาณการเพิ่มจำนวนของเซลล์ได้ด้วยเทคนิค MTT assay พบว่า โอพีเอ็นไม่ส่งเสริมกระบวนการเพิ่มจำนวนของเซลล์ โดยสรุปแล้วงานวิจัยนี้ได้แสดงถึงศักยภาพในการนำโอพีเอ็น ไปประยุกต์ใช้ทางด้านวิศวกรรมเนื้อเยื่อกระดูก

ภาควิชา	เคมี	ลายมือชื่อนิสิต	.....
สาขาวิชา	เคมี	ลายมือชื่อ อ.ที่ปรึกษาหลัก	.....
ปีการศึกษา	2559	ลายมือชื่อ อ.ที่ปรึกษาร่วม	.....

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PANITTHA DAMSONGSANG: OPN IMMOBIZATION ON SURFACE-GRAFTEDPOLY(ACRYLIC ACID)BRUSHES TO PROMOTE OSTEOBLAST ADHESION AND PROLIFERATION. ADVISOR: ASSOC. PROF. VORAVEE HOVEN, Ph.D., CO-ADVISOR: PROF. PRASIT PAVASANT, Ph.D., 43 pp.

In this research, glass substrates were grafted with poly(acrylic acid) (PAA) brushes via surface-initiated reversible addition-fragmentation chain transfer (RAFT) polymerization. ^{1}H NMR analysis of PAA formed in solution indicated that the polymerization was well-controlled. Osteopontin (OPN) synthesized from Tobacco plant was then immobilized on the surface-grafted PAA brushes (SiO_2/Si-OPN) using EDC/NHS as coupling agents. Stepwise surface modification was verified by water contact angle measurements, Fourier transform-infrared spectroscopy (FT-IR), x-ray photoelectron spectroscopy (XPS) and atomic-force microscopy (AFM). Quantification of OPN immobilized on surface was determined by ELISA assay. The percentages of OPN immobilization efficiency were higher than 95% at all concentration tested. MC-3T3-E1 cells cultured on the SiO_2/Si-OPN were better spreading than those on pristine glass substrate and gelatin-modified surface. Moreover, polymerase chain reaction (PCR) analysis indicated that expression levels of the following genes, namely collagen I (Col I), Osterix (Osx), Runt-related transcription factor 2 (Runx2) of MC-3T3-E1 cells on the SiO_2/Si-OPN were higher as compared with those on pristine glass substrates and gelatin-modified surface. As determined by MTT assay, OPN could not promote cell proliferation. These results indicated that OPN immobilization on surface-grafted poly (acrylic acid) brushes can promote osteoblast adhesion and differentiation implying that OPN can potentially be used for bone tissue engineering applications.

Department:	Chemistry	Student's Signature	.....
Field of Study:	Chemistry	Advisor's Signature	.....
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LIST OF ABBREVIATION

AA	: Acrylic acid
ACVA	: 4,4′-Azobis(4-cyanovaleric acid)
AFM	: Atomic-force microscopy
ALP	: Alkaline phosphatase
APTES	: 3-Aminopropyltriethoxysilane
COL1	: Collagen type 1
CPPA	: 4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid
CTA	: Chain transfer agent
DCC	: Dicyclohexylcarbodiimide
DMAP	: 4-Dimethylaminopyridine
DMF	: Dimethylformamide anhydrous
DMSO	: Dimethyl sulfoxide
DP	: Degree of polymerization
cDNA	: Complementary deoxyribonucleic acid
EDC	: 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
ELISA	: Enzyme-Linked Immunosorbent Assay
FTIR	: Fourier-Transform Infrared Spectroscopy
GAPDH	: Glyceraldehyde-3-phosphate dehydrogenase
^{1}H-NMR	: Proton nuclear magnetic resonance
MTT	: (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NHS	: N-hydroxysuccinimide

OSX	: Osterix
PBS	: Phosphate buffered saline
PAA	: Poly(acrylic acid)
RAFT	: Reversible addition-fragmentation chain transfer
RNA	: Ribonucleic acid
RUNX2	: Runt-related transcription factor 2
XPS	: X-ray photoelectron spectroscopy
qPCR	: Quantitative real-time polymerase chain reaction

CHAPTER I
INTRODUCTION

1.1 Introduction

Tooth loss is a major problem in elder patients. It is often accompanied by jawbone loss. Dentures are usually employed. Weakness may be suffered if the dentures are placed with insufficient bone volume. One solution to solve this problem is the use of dental implant [1] to support dentures for improving stability that would result in the efficient occlusion. However, the success of implantation is questionable in elder patients due to their insufficient number of stem cells and delay or impaired healing. Consequently, the implants are not properly fit and have short lifespan [2]. One of the ways for developing the implant surface is to immobilize biomolecules or proteins to increase the implant efficiency.

Examples of research involve the immobilization of biomolecules or proteins on the various surface for cell applications.

In 2009, Raynor *et al.* [3] grafted poly(2-gluconamidoethyl methacrylate) (poly(GAMA)) onto titanium surface via surface-initiated atom-transfer radical polymerization (SI-ATRP). GFOGER-containing peptide sequence was then immobilized on poly(GAMA). They found that the modified titanium surface with GFOGER peptide can promote MC-3T3-E1 spreading better than unmodified titanium surfaces.

In 2011, Ren *et al.* [4] has modified the surface of titanium to stimulate the growth of bone cells. They grafted poly (oligo (ethylene glycol) methacrylate-*r*-hydroxyethyl methacrylate) (poly(OEGMA-r-HEMA)) copolymer onto the surface through surface-initiated polymerization. The surface-grafted polymer then reacted with succinic anhydride via carboxylation in order to change hydroxyl groups of the copolymer to carboxylic groups and was immobilized with Fibronectin (FN) and recombinant human bone morphogenetic protein-2 (rhBMP-2) inhibitors, which promoted the adhesion of MC3T3 cells on the surface and also increased alkaline phosphatase (ALP) gene expression.

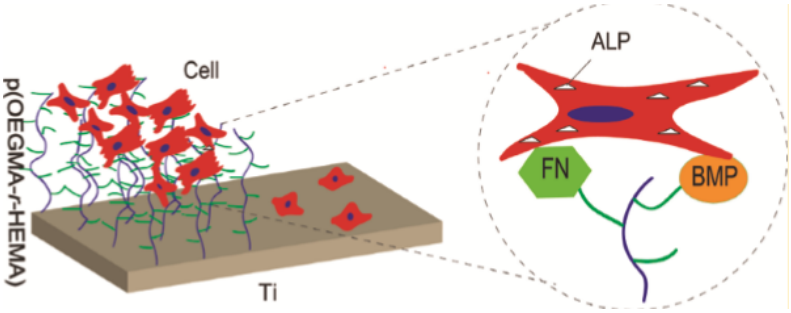

Figure 1.1 Ti surface modified with poly(OEGMA-r-HEMA) and immobilized with Fibronectrin (FN) and recombinant human bone morphogenetic protein (rhBMP-2). [4]

In 2016, Lee *et al.* [5] prepared the three-dimensional polycaprolactone (PCLSs) scaffold grafted with polydopamine (DOPA) and treated with 100 ng/ml of rhBMP2 (PCLSD 100) and 500 ng/mL of rhBMP2 (PCLSD 500) and then tested for cell applications by culturing MC3T3-E1 on the surface. They found that cell proliferation and ALP activity of the PCLSD 500 were greater than those of the PCLSs and PCLSD100. Moreover, calcification of MC3T3-E1 cultured on the PCLSD was verified by alizarin red assay. They found that PCLSD 500 had higher calcium deposition than other samples. These results implied that this surface modification can accelerate differentiation of MC3T3-E1.

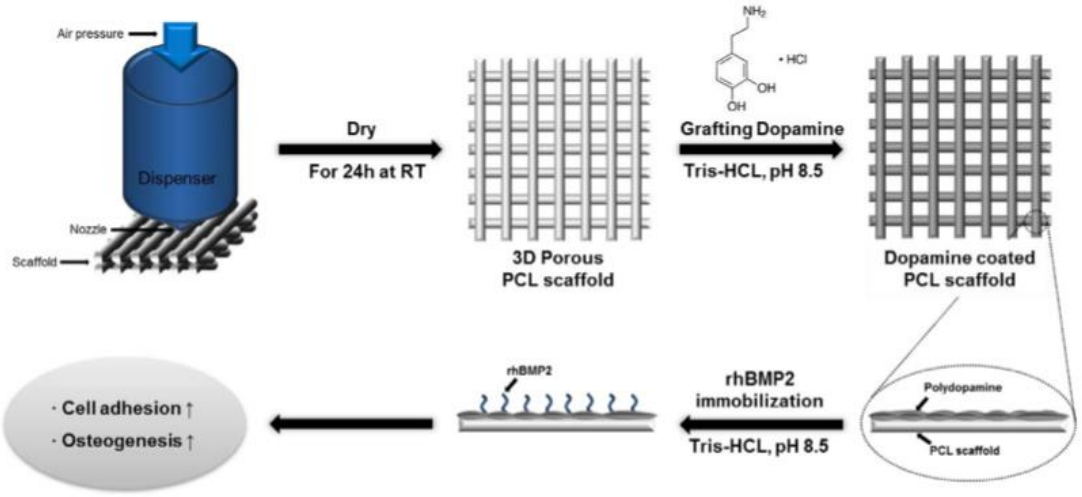

Figure 1.2 Polycaprolactone (PCLSs) scaffold grafted with polydopamine (DOPA) and treated with rhBMP2 for cell application testing. [5]

In 2014, Kim *et al.* [6] modified the titanium surface by grafting heparin which is polysaccharide having glycosaminoglycan repeating units with abundant of sulfate groups. Heparin is recognized as biomolecule that can capture various growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) were grafted on titanium surface via amide bond formation using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), *N*-hydroxysuccinimide (NHS) to activate the carboxyl groups of heparin resulting in BMP-2/Ti. Then, bone morphogenetic protein-2 (BMP-2) which can promote differentiation of mesenchymal stem cells (MSCs) to osteoblast cells were immobilized on the titanium surface modified with heparin by electrostatic interaction between the negative charge of the heparin and the positive charge of BMP-2 and yielded BMP-2/Hep-Ti. MG-63 cells proliferation on the BMP-2/Hep-Ti was greater than those of Ti and BMP-2/Ti. The increasing of ALP activity, calcium deposition including osteocalcin (OCN) and osteopontin (OPN) levels compared to Ti and BMP-2/Ti implied that BMP-2/Hep-Ti can promote differentiation of osteoblast cells [7] more efficiently.

Osteopontin (OPN) is extracellular matrix [8] glycosylated phosphoprotein which has various functions [9, 10] in the wide range of cells [11]. Molecular weight of OPN is 32 kDa and increase from 44 to 75 kDa after post-translation modifications by

phosphorylation and N-linked glycosylation.[9, 12]. As previously reported, OPN can promote endothelial cell adhesion to the environment and angiogenesis [13] which is mediated by arginine-glycine-aspartic acid (RGD) containing domain, important amino sequence for specific binding to $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_1$ integrin on the cells [10, 13], having the role especially on osteoblast differentiation [14], new bone formation [15] and bone mineralization [16].

OPN extracted from Tobacco, which were N-glycosylated to mimic N-glycosylated proteins in the human body [17], Plant-derived OPN was expressed in *Nicotiana benthamiana* leaves using geminiviral vector and purified by Ni affinity chromatography. The purified OPN was characterized by Western blot using anti-OPN antibody showing that the molecular weight of plant-derived OPN was 50kDa. [18] **(Figure 1.3)**.

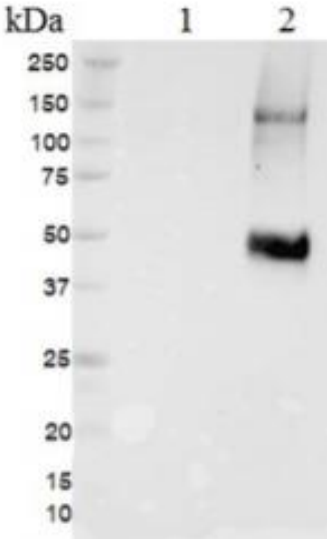

Figure 1.3 Western blot of plant-derived OPN [18]**.**

There are researches reporting on properties of OPN for cell applications.

In 2011, Jensen *el al.* [15] used hydroxyapatite (HA) particles functionalized with OPN for covering on the canine endosseus gap implant model made of poly-D, L-lactic acid (PDLLA) for 4 weeks in media for inducing new bone formation. It was found that HA functionalized with OPN increased the formation of new bone.

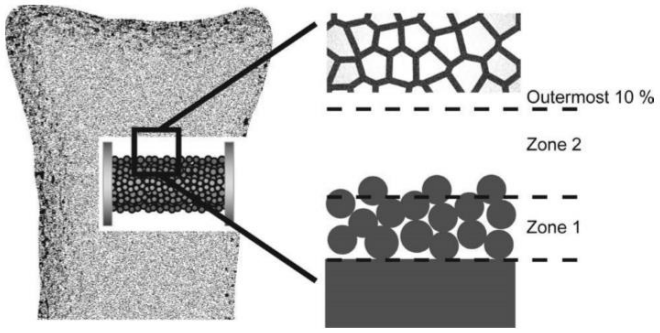

Figure 1.4 The canine endosseus gap implant model of hydroxyapatite (HA) particles functionalized with OPN. [15]

Examples of research have been reported on OPN immobilization on various surfaces.

In 2003, Martin *et al.* [13] prepared film of poly(2-hydroxyethyl methacrylate) (polyHEMA) which has hydroxyl group repeating units for OPN immobilization using 1,1'-carbonyldiimidazole (CDI) as a coupling agent via amide bond. They found that OPN-immobilized on poly(HEMA) could promote bovine endothelial cell adhesion.

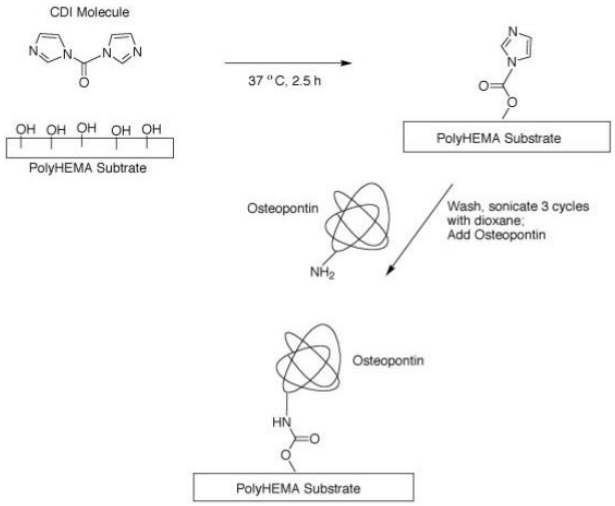

Figure 1.5 Poly(HEMA) immobilized with OPN using CDI as coupling agent [13]

In 2014, Kim *et al.* [6] prepared a film of polycaprolactone (PCL) grafting with 1,6-hexanediamine having amino end groups (aminated PCL). Collagen cross-linked with OPN (col-I-OPN) were then immobilized on aminated PCL via amide bond. Human Adipogenic Mesenchymal Stem Cell (hADMSC) were cultured on both modified and unmodified surface. They found that hADMSC attachment and proliferation were greater on the modified PCL than the unmodified PCL.

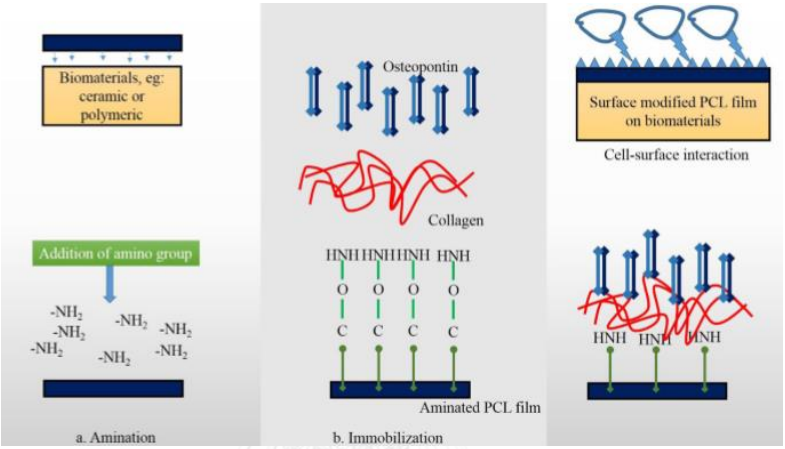

Figure 1.6 Scheme of the immobilization of collagen-osteopontin on aminated PCL surface. [6]

Polymer brushes are polymer chains having their ends tether to the surface by covalent bond [7]. Polymer brushes can be prepared by either surface-initiated polymerization or "grafting from" approach or "grafting onto" approach. These methods provide higher stability than physical adsorption and molecular weight and chain length can be manipulated using well-controlled polymerization processes [19-21]. Polymer brush chains have high functional groups per surface area in a higher proportion than the self-assembled monolayers of thiols or silane compounds which are often used to modify the surface functional groups [21, 22] For this reason, the surface-grafted polymer provide higher efficiency for immobilization of biomolecules and proteins.

In recent years, poly(acrylic acid) (PAA) brushes have been widely used for protein immobilization due to the abundant of carboxyl groups, the repeating units of PAA brushes chains, can effectively immobilize biomolecules or proteins on various surface. Many research works have been reported on poly (acrylic acid) brushes grafted on various surface in order to increase the effective of protein immobilization.

In 2012, Audouin *et al.* [23] grafted poly(acrylic acid) (PAA) brushes on the macroporous polymers synthesized in high internal phase emulsions (PolyHIPE) surface by activator regeneration by electron transfer-atom transfer radical polymerization (ARGET-ATRP) of tert-butyl acrylate in order to prepared poly(t-butyl acrylate). t-Butyl groups were then deprotected to convert poly(t-butyl acrylate) to poly(acrylic acid) brushes. The material was then immobilized with green fluorescent protein (eGFP) and coral-derived red fluorescent protein (DsRed) by activating carboxyl groups of PAA brushes using EDC/sulfo-NHS as coupling agent. It is anticipated that these materials are very useful for bioseparation applications.

In 2013, Qu *et al.* [22] grafted PAA brushes onto silica particles via a reversible addition-fragmentation chain transfer (RAFT) polymerization. It was found that RAFT polymerization provided well-controlled molecular weight and thickness. Then, streptavidin (SA) proteins which can specifically bind biotin were immobilized on PAA brushes via amide bond by activating the carboxyl groups of PAA using EDC/NHS. They found that the thickness of PAA brushes on particle was 14.6-68.8 nm containing carboxyl groups of 0.82-2.37 mmol/g. The protein immobilization capacity was 2,600 μg of SA per 1 mg of silica particles. It was found that PAA brushes could provide SA immobilization on silica particles 60-folds higher than the conventional functionalized monolayer method.

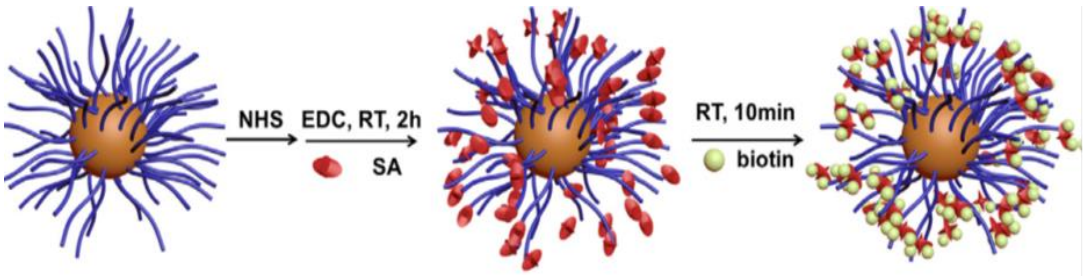

Figure 1.7 Immobilization of streptavidin (SA) for biotin binding on silica particles. [22]

In 2013, Wang *et al.* [24] prepared protein patterning by microcontact printing (μCP) method and then grafted PAA brushes on gold surface. Human immunoglobulin G (H-IgG) and goat anti-H-IgG (G-H-IgG), which were antigen and antibody in the body respectively, were then immobilized on PAA brushes via amide bond. This modified materialis very useful for biosensor or protein microarrays.

In 2011, Akkahat *et al.* [21] prepared poly(*tert*-butyl acrylate) (P*t*-BA) brushes by surface-initiated atom transfer radical polymerization. *Tert*-butyl groups were then removed by acid hydrolysis to yield PAA brushes which were used as a three dimensional (3D) precursor layer for biosensing applications. Biotin as model bioactive probe was immobilized on the PAA brushes. The efficiency of bioactive probe was determined by the binding of streptavidin (SA) with biotin-attached PAA brushes. The increasing of the signal for biospecific binding of SA on biotin-attached PAA brushes as compared to a self-assembled monolayer (SAM) of a carboxyl-terminated alkanethiol which was used as a model for two-dimentional (2D) precursor layer implied that the PAA brushes can be effectively used for the development of biosensors.

This research aims to test the ability of OPN extracted from Tobacco as bioactive compound to promote osteoblast adhesion and differentiation. Glass substrate was used as a model platform to immobilize this plant-derived OPN. Poly(acrylic acid) (PAA) brushes was first grafted on the glass substrate by surface-initiated polymerization of acrylic acid using a well-controlled polymerization based on reversible addition fragmentation chain transfer (RAFT) mechanism. It was anticipated that the abundantly available carboxyl groups of PAA brushes would provide multiple functional groups for OPN immobilization via amide bond formation. Biological responses of the surface-

immobilized OPN were tested against human osteoblasts in terms of adhesion, proliferation and gene expression.

Moreover, we expected that the results from this research can be used as a guideline to modify the surface of the other materials such as dental implant, guided tissue regeneration (GTR) membrane or scaffold in order to develop biomedical materials for promoting osteoblast adhesion, proliferation and differentiation. Since OPN extracted from Tobacco can be economically produced in large scale and short period of time in comparison with commercial OPN form human or mammalian, it is possible to develop this plant-derived OPN for commercial use in the future.

1.2 Objective

1. To prepare and characterize glass substrates having surface-grafted poly (acrylic acid) brushes and immobilized with plant-derived OPN.

2. To determine biological responses of osteoblasts on glass substrates having surface-grafted poly(acrylic acid) brushes and immobilized with plant-derived OPN.

1.3 Scope of investigation

1. Literature survey for related work.

2. Preparation and characterization of glass substrates having surface-grafted poly(acrylic acid) brushes prepared by surface-initiated RAFT polymerization.

3. Immobilization of plant-derived OPN on the glass substrate having surface-grafted poly(acrylic acid)

4. Characterization of surface-modified substrates after stepwise modification using water contact angle analysis, FT-IR spectroscopy, AFM and XPS analysis

5. Determination of the amount of immobilized OPN per surface area and OPN immobilization efficiency by Human OPN ELISA assay

6. Determination of adhesion/proliferation of human osteoblasts on the glass substrates having surface grafted with poly(acrylic acid) and immobilized with plant-derived OPN by MTT assay.

7. Determination of the morphology of human osteoblasts on the glass substrate having surface-grafted poly(acrylic acid) and immobilized with plant-derived OPN

8. Determination of differentiation of human osteoblasts cultured on the glass substrate having surface-grafted poly(acrylic acid) and immobilized with plant-derived OPN by real time-quantitative polymerase chain reaction (qPCR)

CHAPTER II

MATERAILS AND METHODS

2.1 Materials

Toluene, acetone, ethanol were purchased from Merck (Germany). Dimethylformamide (DMF) was purchased from RCI Labscan (Thailand). 3-Aminopropyltriethoxysilane (APTES), 4-dimethylaminopyridine (DMAP), dicyclohexylcarbodiimide (DCC), 4,4′-azobis(4-cyanovaleric acid) (ACVA), 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid (CPPA) (Chain transfer agent or CTA) and *N*-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich (USA). Acrylic acid (AA) supplied by Aldrich was purified by vacuum distillation. *N*-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) was purchased from TCI chemicals (Japan). Sulfuric acid (H_2SO_4) and hydrogen peroxide (H_2O_2) were purchased from Merck (Germany). Glass coverslips (i.d.=18 mm) were supplied by S.E. SUPPLY LTD PART (Thailand). OPN synthesized from Tobacco was supplied by Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Science, Chulalongkorn University.

2.2 Preparation of surface grafted initiator (SiO_2/Si-ACVA)

Glass coverslips were cleaned with a Plasma Cleaner (Rame´-Hart, Inc., USA, model 100-00) for 5 minutes on both sides. Glass sildes were then silanized via vapor silanization with 200 µL of APTES in a closed vial in an oven at 80 °C for 72 hours and then rinsed with toluene, acetone, DI water and dried with nitrogen gas respectively to give SiO_2/Si-APTES **(Figure 2.1, Step I**). ACVA (0.21 g, 1 mmol,.) as initiator, DCC (0.19 g, 1 mmol.) and DMAP (9.19 mg, 0.1 mmol) as coupling reagents were dissolved in 20 mL of DMF. The solution was stirred under nitrogen atmosphere at room temperature for 4 hours and then transferred to a glass tube containing SiO_2/Si-APTES and equipped with a magnetic stirred bar under nitrogen gas. After 20 hours of reaction, the SiO_2/Si-APTES were rinsed with DMF, ethanol and dried with nitrogen gas and yielded glass substrates functionalized with initiator (SiO_2/Si-ACVA) (**Figure 2.1, Step II**).

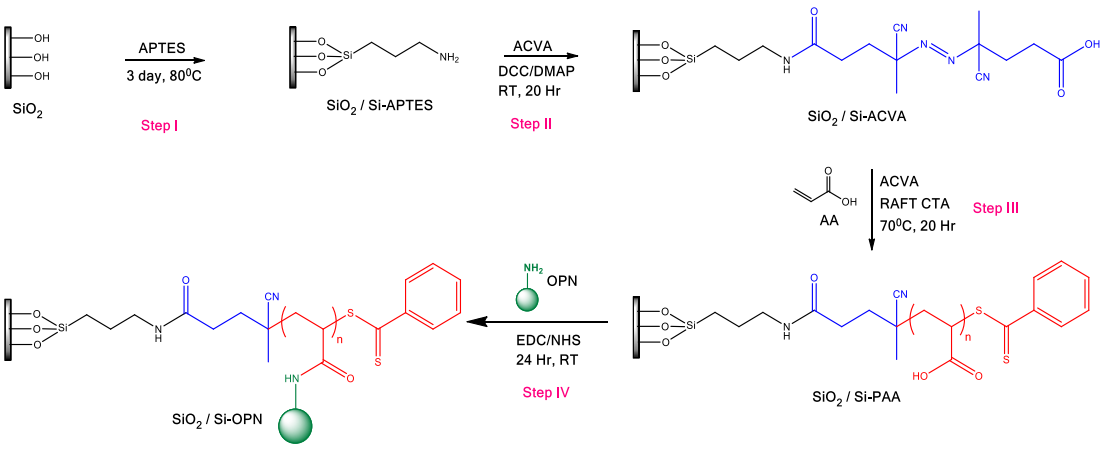

Figure 2.1 Preparation of PAA-grafted substrates and subsequent OPN immobilization

2.3 Preparation of surface grafted with PAA brushes (SiO_2/Si-PAA)

Surface-initiated RAFT polymerization of AA was performed by firstly introducing a solution of CTA (0.056 g, 0.2 mmol), ACVA (0.014 g, 0.05 mmol) and AA (1 M, 1.37 mL) in 18 mL of MilliQ mixed with 2 mL of phosphate buffer saline (PBS) in a glass tube containing SiO_2/Si-ACVA and equipped with a magnetic stirred bar. After the reaction proceeded under nitrogen atmosphere at 70 °C for 20 hours, the obtained SiO_2/Si-PAA were rinsed with ethanol, DI water and dried with nitrogen gas (**Figure 2.1, Step III**).

2.4 OPN immobilization on glass surface (SiO_2/Si-OPN)

The SiO_2/Si-PAA substrates were activated at room temperature for 30 minutes by using 0.2 M of EDC and 0.05 M of NHS as coupling agents in MilliQ water. Then, the EDC/NHS solution was removed and the substrates were rinsed three times with MilliQ water. After surface activation, the SiO_2/Si-PAA substrates were incubated with varied concentration of OPN solution (0.06-30 ng/mL) in PBS buffer with shaking at room temperature for 24 hours. Finally, the glass substrates immobilized with OPN (SiO_2/Si-OPN) were then rinsed five times with PBS and dried with nitrogen gas (**Figure 2.1, Step IV**).

2.5 Preparation of surface-functionalized silica particles for FT-IR characterization

Piranha solution (H_2SO_4: H_2O_2 = 7: 3) was prepared by slowly dropped with 7 ml of H_2SO_4 into 3 ml of H_2O_2. The solution was then mixed with silica particles (0.3 mg) in 50 mL glass flask for 1 hour to clean the silica particles. Then, silica particles are filtered and washed with DI water several times until the water were no longer acidic (pH =7) tested by universal indicator. The cleaned silica particles were dried in an oven at 120℃ for 2 hours, then the cleaned silica particles were silanized by adding 2% v/v APTES in 10 ml of toluene equipped with a magnetic stirred bar under nitrogen gas for 18 hours. The silica particles were then vacuum filtered and washed with toluene, acetone and dried by dried in an oven at 120 ° C for 1 hour. After that, ACVA (0.11 g, 1 mmol,.) as initiator, DCC (0.10 g, 1 mmol.) and DMAP (4.59 mg, 0.1 mmol) as coupling reagents were dissolved in 10 mL of DMF under nitrogen atmosphere at room temperature for 4 hours and then transferred to a glass tube containing silica particles functionalized with APTES, equipped with a magnetic stirred bar and purged with nitrogen gas. After 20 hours of reaction, the functionalized silica particles were then vacuum filtered and rinsed with DMF, ethanol to yield initiator-functionalized silica particles. The initiator-functionalized silica particles were grafted with PAA brushes and subsequently immobilized with OPN on following the procedure in 2.3 and 2.4, respectively.

2.6 Characterization

The molecular weight of PAA solution was determined by ^{1}H-NMR recorded in D_2O using a Varian NMR spectrophotometer, model Mercury-400 nuclear magnetic resonance spectrometer (USA) operating at 400 MHz. The dynamic advancing and receding water contact angles were measured using a contact angle goniometer, model 100-00, equipped with a Glimont syringe and a 24-gauge flat-tipped needle (Ramé-Hart, Inc., USA). AFM images of surface-modified silicon wafers were recorded with Scanning Probe Microscope model NanoScope® IV, Veeco, USA. Measurements were performed in air using tapping mode. Silicon nitride tip with a resonance frequency of 267-295 KHz and a spring constant 20-80 N/m were used. The FT-IR spectra of surface-modified silica particles were recorded with a FT-IR spectrometer (Perkin Elmer), model system 2000, with 32 scans at a resolution of 4 cm^{-1} using a TGS detector. The stepwise surface modification was semi-quantitatively determined by XPS on AXIS Ultra DLD spectrometer (Kratos Analytical Ltd., Manchester, England).

2.7 Quantification of surface-immobilized OPN by ELISA assay

The quantity of OPN immobilized on the surface was indirectly determined from the remaining of OPN in the supernatant collected after immobilization by ELISA assay, with the procedure taken from manuals of Human Osteopontin (OPN) ELISA Kit (Sigma-Aldrich, USA). First, the collected supernatant was lyophilized (model Freezeone 77520 Benchtop, Labconco, USA) to obtain the remaining OPN. The obtained OPN was then dissolved in 300 µl of diluent buffer B and incubated in ELISA Kit well for 24 hours at 4°C with gentle shaking. After incubation, the solution was removed and the well was rinsed 4 times with 300 µl of 1X washing buffer. Then, 100 µl of 1X prepared biotinylated detection antibody was added to each well and incubated for 1 hour at room temperature with gentle shaking followed by removal of solution and washing with 300 µl of 1X wash buffer. Next, 100 µl of ELISA colorimetric TMB reagent was added to each well and incubated for 30 minutes at room temperature in the dark with gentle shaking. Finally, 50 µl of stop solution was added to each well. The absorbance at 450 nm was immediately measured using UV/Vis microplate reader.

2.8 Determination of cellular responses

MC-3T3-E1 cells were cultured on glass substrates for 3 hours at 50,000 cells/well on glass substrates (i.d. 1.8 mm) in 12-well plates as a monolayer in HyClone Minimum Essential Medium (MEM) with Earle's Balanced Salts (MEM/EBSS; GE healthcare, Logan, Utah, USA) supplemented with 10% fetal bovine serum, 1% L-glutamine. The cultures were maintained at 37°C with 5% CO_2. Then, the cells were fixed with 500 µL/well of 3.7% formaldehyde for 15 minutes and then added 0.1% triton-X-100 (USB Corporation Cleveland, OH USA) for 3 minutes to permeabilize cell membrane. After cell fixation, the cells were incubated in 1% BSA (SIGMA-ALDRISH Co., St. Louis, MO USA) in PBS for reducing nonspecific background. The fixed cells were sequentially incubated with 1:200 of Phalliodin-Rhodamine conjugated (Invitrogen; Thermo Fisher Scientific, MA USA) and 1:5000 of DAPI for 20 minutes each to stain actin and nucleus, respectively. Cell morphology was observed by a ZEISS Observer.Z1 fluorescent microscope.

2.9 Gene expression analysis by real time-quantitative polymerase chain reaction (qPCR)

MC-3T3-E1 cells were cultured on glass substrates for 3 hours at 300,000 cells/well on glass substrates in 12-well plates as a monolayer in HyClone Minimum Essential Medium (MEM) with Earle's Balanced Salts (MEM/EBSS; GE healthcare, Logan, Utah, USA) supplemented with 10% fetal bovine serum, 1% L-glutamine, 50 µg/ml of ascorbic acid, 5 mM of β-glycerophosphate and 2.5 µM of dexamethasone. The cultures were maintained at 37°C with 5% CO_2. RNA was extracted with 1 mL Trizol reagent and quantified by using a NanoDrop 2000 spectrophotometer (Thermo scientific, Wilmington, DE USA). RNA (1 ng) was conversed to cDNA with Reverse transcriptase enzyme by ImProm-II RT (Promega, Madison, WI USA). After conversion process, cDNA underwent the qPCR reaction by a LightCycler instrument (Roche Diagnostics, USA) with the LightCycler 480SYBR Green-I Master Kit. Gene expression was calculated by RelQuant software (Roche Diagnostics, USA) and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Forward and reverse

sequencing primer of following genes namely, GAPTH, Collagen type I, Osterix (Osx) and Runt-related transcription factor 2 (Runx2) are shown in **Table 2.1**.

Table 2.1 Forward and reverse sequencing primer of the marker genes for qPCR

Genes	forward sequencing primer (5'–3')	reverse sequencing primer (5'–3')
GAPDH	CAC TGC CAA CGT GTC AGT GGT G	GTA GCC CAG GAT GCC CTT GAG
Col I	TGT CCC AAC CCC CAA AGA C	CCC TCG ACT CCT ACA TCT TCT GA
Osx	CTG GTC TGA CTG CCT GCC TAG	GCG TGG ATG CCT GCC TTG TA
Runx2	CGG GCT ACC TGC CAT CAC	GGC CAG AGG CAG AGG TCA GA

2.10 Determination of cell adhesion and proliferation by MTT assay

MC-3T3-E1 cells were cultured on glass substrates for 3 hours at 50,000 cells/well on glass substrates in 12-well plates as a monolayer in HyClone Minimum Essential Medium (MEM) with Earle's Balanced Salts (MEM/EBSS; GE healthcare, Logan, Utah, USA) supplemented with 10% fetal bovine serum, 1% L-glutamine. The cultures were maintained at 37°C with 5% CO_2. MTT assay was used to investigate cell adhesion and proliferation (Thiazolyl blue, Sigma, MO, USA). After 2 hours, 1, 2 and 3 day, the media solution was removed, and 0.5 mg/ml MTT/normal saline solution without phenol red was added to the well plate and incubated under 5% CO_2 at 37 °C for 15 minutes. Then the solution was removed and 1 ml of Glycine buffer mixed with DMSO in ratio 1:9 was added to dissolve the purple crystals of formazan. The measurement was done by microplate reader at the wavelength of 570 nm. The cell proliferation ratio was calculated using **Equation 2.1**. OD control and OD sample is defined as optical density of pristine glass surface and the sample, respectively.

$$\%\ cell\ viability = \frac{OD_{sample}}{OD_{control}} \times 100 \qquad \textbf{Equation 2.1}$$

CHAPTER III

RESULTS AND DISCUSSION

3.1 Preparation of PAA via RAFT polymerization

PAA were formed via RAFT polymerization at 70 ° C for 20 hours as shown in **Figure 3.1** The crude PAA solution was purified by dialysis in DI water for 72 hours that provided the pure PAA solution. PAA formed in solution were characterized by ^{1}H-NMR spectroscopy as shown in **Figure 3.2**. An appearance of signals from aromatic protons (peak a) in ^{1}H-NMR spectra suggested the presence dithiobenzoate group at the chain end of PAA. The fact that the signals of vinylic protons (peak b and c) from acrylic acid disappear from the ^{1}H-NMR spectrum of pure PAA indicates that all residual acrylic acid was effectively removed after purification.

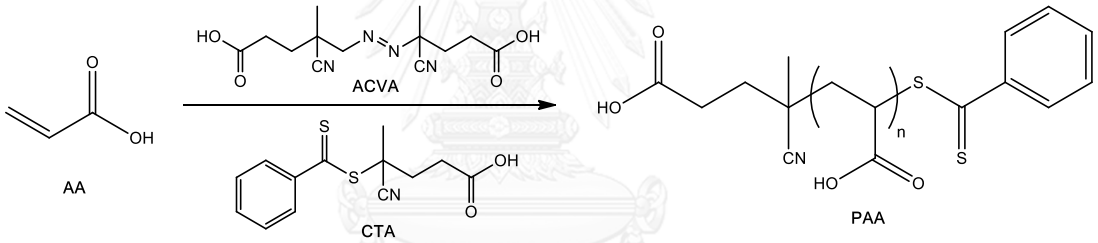

Figure 3.1 Scheme of PAA formation in solution via RAFT polymerization.

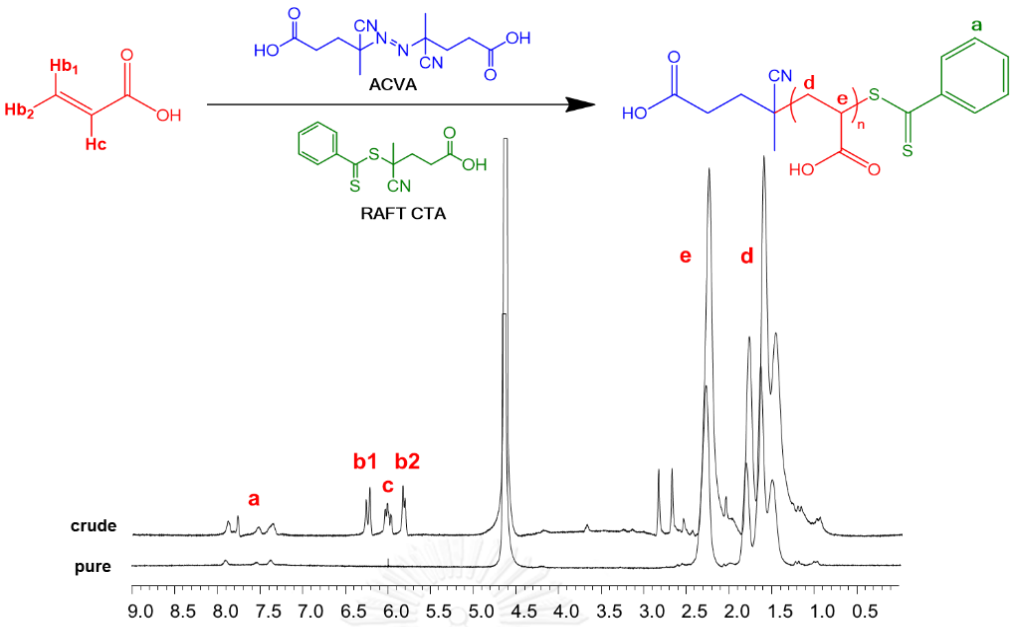

Figure 3.2 ^{1}H-NMR spectra of PAA formed in the solution.

% Conversion was calculated by the relative ratio between the peak integration of acrylic acid monomer (peak b1, b2 and c) and the peak integration of PAA backbone (peak e and d) as shown in **Equation 3.1**.

It was found that the calculated conversion value is 97.1%. Moreover, the M_n of PAA was calculated by relative ratio between the peak integration of PAA backbone (peak e) and the peak integration of dithiobenzoate group (peak a) as shown in **Equation 3.2**. The calculated molecular weight was found to be 7,728. This value closely resembles that of theoretical molecular weight (7,206) suggesting that the RAFT polymerization is well controlled.

$$\%\ Conversion = \left\{\frac{integral\ of\ H_{b,c}}{integral\ of\ H_{e,d}}\right\} \times 100 \qquad \textbf{Equation 3.1}$$

$$Average\ M_n = \left\{ \frac{integral\ of\ H_e \times\ M_{n(acrylic\ acid)}}{\left(\frac{integral\ of\ the\ H_a}{5}\right)} \right\} +\ M_{n\ (CTA)}$$

M_n (acrylic acid) = 72.06 g/mol, M_n (CTA) = 279.38 g/mol **Equation 3.2**

3.2 Preparation and characterization of PAA-grafted substrates

The 4,4'-azobis (4-cyanovaleric acid) (AVCA) used as initiator has to be grafted on the glass surface in order for the substrate to be able to initiate polymerization from its surface. However, the bonding between the inorganic compound (glass substrate) and the organic compound (ACVA) is unstable. It is necessary to first react the silanol groups of the clean glass substrate with a silane compound, 3-aminopropyl triethoxysilane (APTES) which yields SiO_2/Si-APTES **(Figure 3.3)**. The amino groups of APTES immobilized on the surface can then form amide bonds with carboxylic groups of ACVA using DCC and DMAP as coupling agents to yield initiator-grafted glass substrate (SiO_2/Si-ACVA) **(Figure 3.4)**. PAA chains were then grown via surface-initiated RAFT polymerization from glass substrate modified with ACVA (SiO_2/Si-ACVA) [7] to yield PAA-grafted glass substrate (SiO_2/Si-PAA) as shown in **Figure 3.5**.

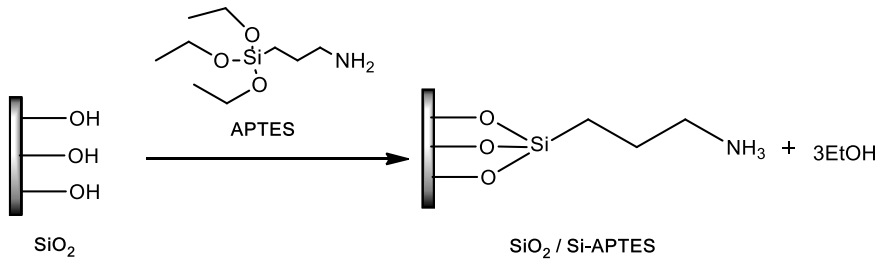

Figure 3.3 Immobilization of APTES on glass substrate by vapor silanization.

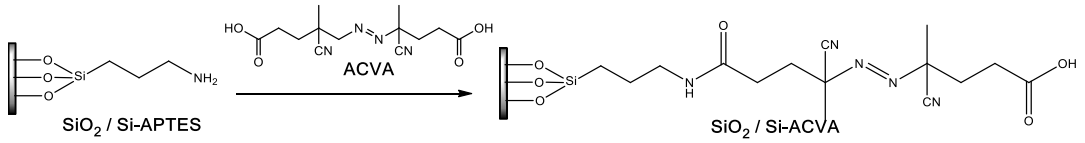

Figure 3.4 Preparation of initiator-grafted glass substrate.

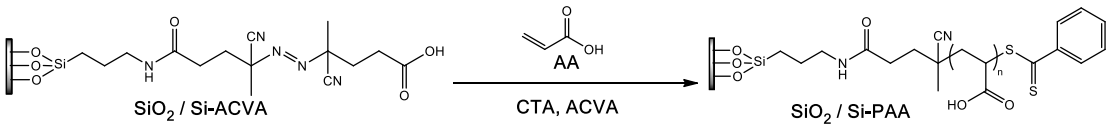

Figure 3.5 Preparation of PAA-grafted glass substrate by surface-initiated RAFT polymerization.

Stepwise surface modification was verified by water contact angle measurements, Fourier transform-infrared spectroscopy (FT-IR) and x-ray photoelectron spectroscopy (XPS). The data of water contact angle measurements shown in **Table 3.1** indicate that the glass substrates became very hydrophilic once grafted with PAA as demonstrated by their water contact angles being much lower than those of SiO_2/Si-ACVA.

Table 3.1 Water contact angle data of surface-modified glass substrates

Sample	Water Contact Angle (°)	
	$\theta_{advancing}$	$\theta_{receding}$
SiO_2	N/A	N/A
SiO_2/Si-APTES	71.3 ± 1.8	52.8 ± 4.5
SiO_2/Si-ACVA	66.7 ± 1.5	35.0 ± 4.5
SiO_2/Si-PAA	24.6 ± 7.8	N/A
SiO_2/Si-OPN	44.7 ± 4.2	9.3 ± 6.8

Chemical functionality was determined by FT-IR analysis of surface-functionalized silica particles undergoing similar chemical modification as the glass substrates. As can be seen in **Figure 3.6**, a characteristic broad peak due to OH-stretching appearing at 3100-3700 cm^{-1} in all spectra indicated the presence of silanol groups. The peaks at 1636 and 1569 cm^{-1} which can be assigned to C=O stretching and N-H bending, respectively emerged in the spectrum of SiO_2/Si-ACVA confirming the success of initiator immobilization via amide bond formation. The characteristic peak at 1723 cm^{-1} in spectrum c indicating C=O stretching of PAA confirming the success of PAA grafting on surface.

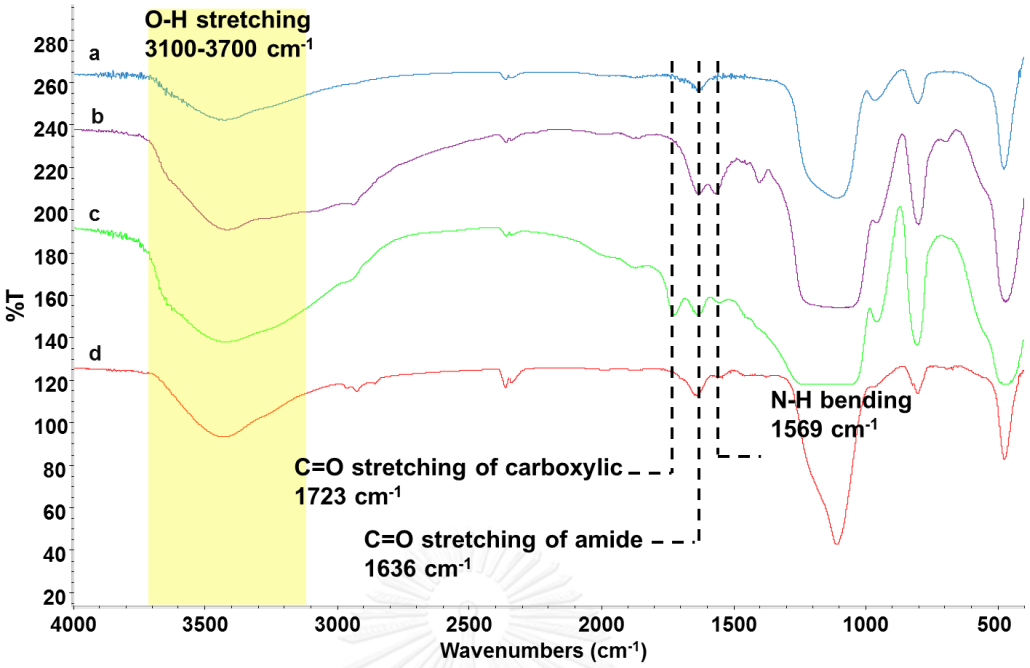

Figure 3.6 FT-IR spectra of a.) SiO_2, b.) SiO_2/Si-ACVA, c.) SiO_2/Si-PAA d.) SiO_2/Si-OPN.

3.3 Preparation and characterization of SiO_2/Si-PAA after OPN immobilization

OPN was immobilized on the surface grafted with PAA brushes (SiO_2/Si-PAA) using EDC and NHS as coupling agents which activated carboxyl groups of PAA into active ester groups. OPN was then immobilized by amide bond formation as shown in **Figure 3.7** The water contact angles went up after OPN immobilization (**Table 3.1**) suggesting that the surface was less hydrophilic because of the increasing of hydrophobicity from carbon backbone of OPN.

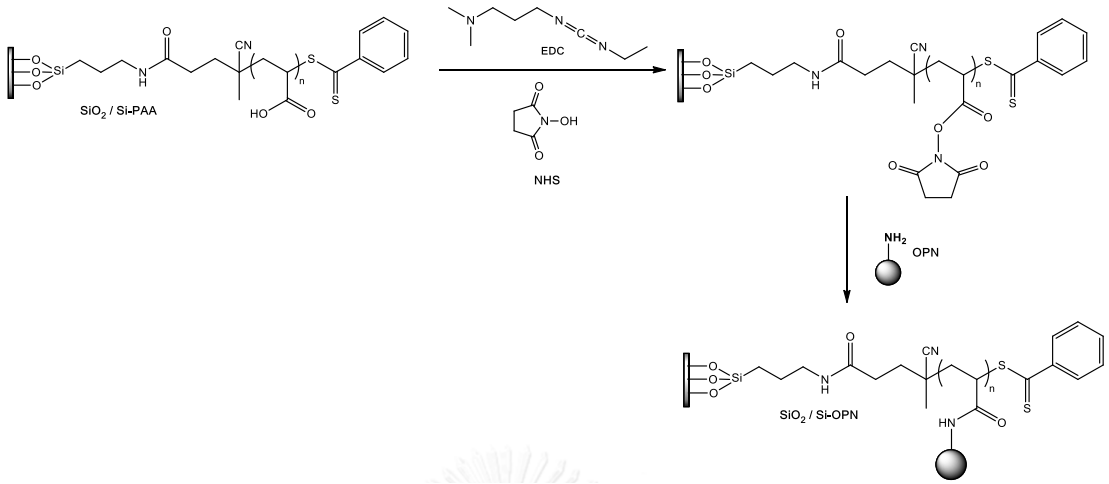

Figure 3.7 Immobilization of OPN on SiO_2/Si-PAA using EDC/NHS as coupling agent.

Moreover, OPN immobilization on the surface-functionalized silica particles can be also confirmed by FT-IR analysis. The spectrum d (**Figure 3.6**) showed the characteristic peak of C=O stretching at 1636 cm^{-1} indicating amide bond formation between carboxyl group of PAA and amino group of OPN. The disappearance of characteristic C=O stretching peak of carboxyl groups in PAA at 1723 cm^{-1} after OPN immobilization strongly suggested that the substrates were modified with OPN.

The success of stepwise surface modification was semi-quantitatively determined by XPS analysis. As tabulated in **Table 3.2**, an increasing % nitrogen from 1.01% of SiO_2/Si-PAA to 3.06, 4.84 and 3.85% for SiO_2/Si-OPN obtained from using OPN having concentration of 0.06, 0.60 and 3.00 ng/mL, respectively for immobilization can be used as an indication of the protein component of OPN on the SiO_2/Si-OPN.

Table 3.2 Element composition (%) determined by XPS of surface-modified glass substrates

Sample	OPN	% element composition			
	(ng/mL)	Si	C	O	N
SiO_2	-	22.68	22.92	54.23	0.17
SiO_2/Si-PAA	-	24.33	26.71	47.95	1.01
SiO_2/Si-OPN	0.06	21.21	29.08	46.65	3.06
SiO_2/Si-OPN	0.6	16.97	37.05	41.13	4.84
SiO_2/Si-OPN	3	17.46	40.42	38.27	3.85

3.4 Surface Morphology by AFM analysis

The surface morphology of the pristine silicon wafer (SiO_2), silicon wafer grafted with PAA brushes (SiO_2/Si-PAA), and silicon wafer grafted with PAA brushes and immobilized with OPN (SiO_2/Si-OPN) having concentration of 3.0, 15.0 and 30.0 ng/mL (OPN 3.0, OPN 15.0 and OPN 30.0 respectively) was analyzed by AFM as shown in **Figure 3.8**. The root-mean-square (RMS) surface roughness of substrate was slightly increased from 0.115 nm of SiO_2 to 0.316 nm of SiO_2/Si-PAA implying that there was a layer of PAA brushes homogeneously and fully covered the substrate. After OPN immobilization, the RMS roughness values went up approximately 5-7 times higher to 1.733, 2.291, 2.137 nm for OPN 3.0, OPN 15.0, and OPN 30.0, respectively indicating the deposition of macromolecules onto the surface. The relatively even distribution of small protrusions (i.d. = 66.7 ± 13.5 nm) strongly suggests that there was protein conjugation [25]. These results also implied that OPN was effectively immobilized on the surface-grafted PAA brushes.

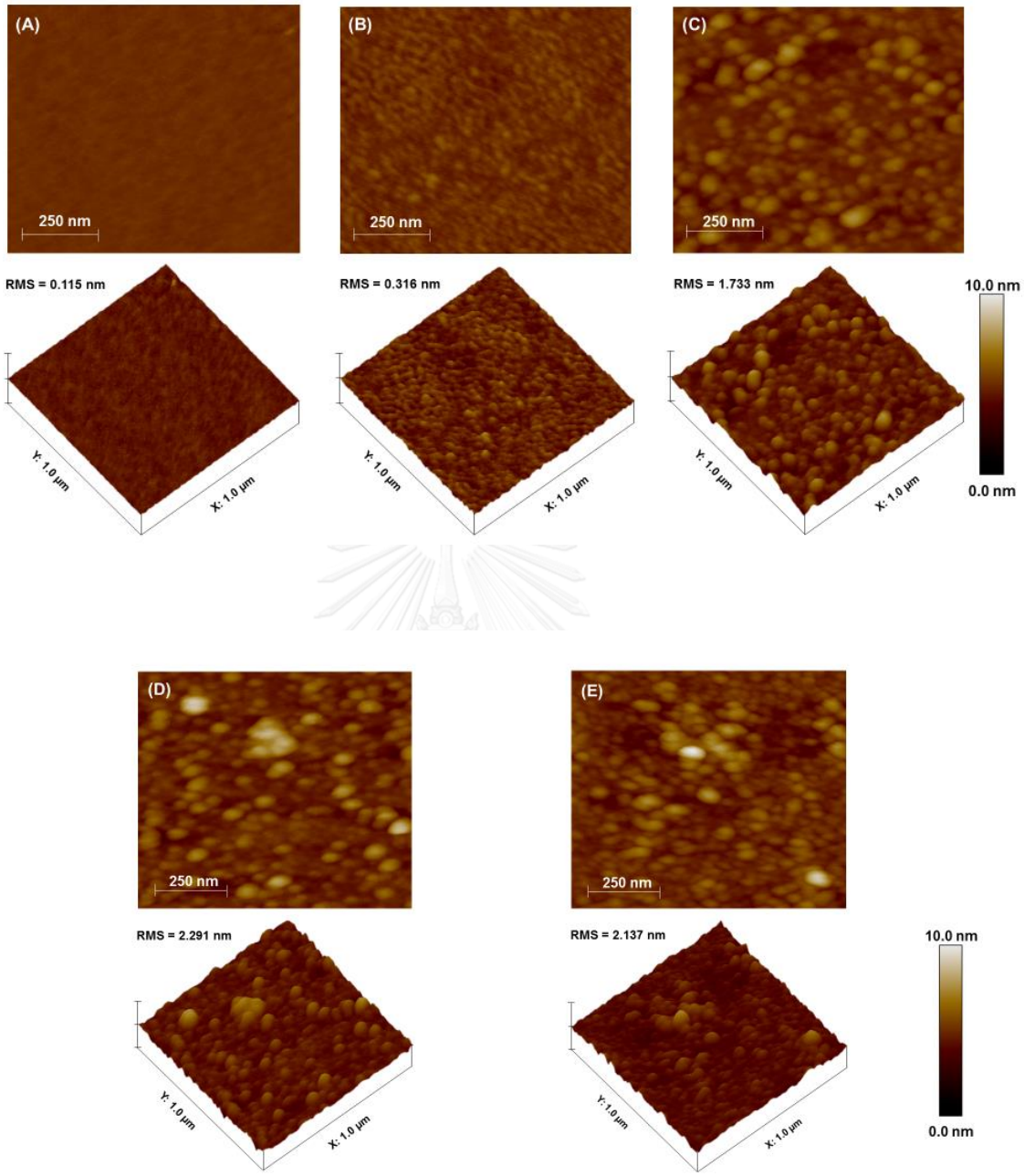

Figure 3.8 AFM morphological images of (A) SiO_2, (B) SiO_2/Si-PAA, (C) OPN 3.0, (D) OPN 15.0, and (E) OPN 30.0.

3.5 Quantification of OPN immobilized on the surface grafted with PAA brushes by ELISA assay

The immobilization efficiency of OPN on the surface grafted with PAA brushes (SiO_2/Si-PAA) was determined by ELISA assay of the supernatant collected after the immobilization step. The amount of OPN can be calculated from standard curve of the relationship between OPN concentration (pg/mL) and absorbance at 450 nm as shown in **Figure A2 (Appendix).** Three independent experiments were performed and the results are concluded in **Table A1 (Appendix).**

Table 3.3 shows the amount of immobilized OPN on the SiO_2/Si-PAA. The amount of OPN immobilized on PAA brushes surface was found to proportionally increase as a function of OPN concentration. The percentages of OPN immobilization efficiency being higher than 95% at all concentration tested strongly suggested that OPN can effectively be grafted on the surface-grafted PAA brushes.

Table 3.3 The amount of immobilized OPN on PAA brushes.

OPN Concentration (ng/mL)	Amount of immobilized OPN (ng) calculated from standard curve	OPN immobilization efficiency (%)	Amount of immobilized OPN on per area (ng/cm^2)*
3.0	3.01 ± 0.01	100.0	0.59-1.18
15.0	14.74 ± 0.20	98.2	2.89-5.78
30.0	29.52 ± 0.34	98.3	5.79-11.58

*The first number and the second number represent the calculated amount of immobilized OPN on one side and both sides, respectively.

3.6 Determination of cellular responses

MC-3T3-E1 cells were cultured in osteogenic media (OM) for 3 hours on SiO_2/Si-OPN prepared from OPN having varied concentration (3.0, 15.0, 30.0 ng/mL). The investigation was done in comparison with pristine glass substrate (SiO_2) and SiO_2/Si-PAA immobilized with varied concentration of gelatin (SiO_2/SiO_2-gelatin) which were used as negative control. Gelatin is derived from collagen [26] which helps to maintain structure of cells [27] and are expected that not to promote cell differentiation. Cell morphology was examined using phase contrast microscope. MC-3T3-E1 cells were treated with phalloidin-Rhodamine and DAPI for staining actin filaments and nucleus, respectively. The micrographs **(Figure 3.9)** and the data shown in **Table 3.4** indicated that MC-3T3-E1 cells cultured on substrates immobilized with bioactive molecules (both OPN and gelatin) spreaded better with larger cell dimension than those on pristine glass and SiO_2/Si-PAA substrates. Moreover, the degree of cell spreading on SiO_2/Si-OPN was apparently superior to that on SiO_2/Si-gelatin suggesting that OPN is more effective in promoting bone cell adhesion.

Table 3.4 % Amount of MC-3T3-E1 cells cultured on surface for 2 hours

Size of cells (μm)	% amount of MC-3T3-E1 cells						
	SiO_2	Gelatin[1] 3.0	Gelatin[2] 15.0	Gelatin[3] 30.0	OPN[4] 3.0	OPN[5] 15.0	OPN[6] 30.0
10-30	23	23	6	2	0	0	1
30-50	67	63	71	69	53	25	32
50-70	9	16	25	29	47	66	60
70-90	4	0	0	1	2	10	8
90-110	0	0	0	0	0	0	1

[1]SiO_2/Si-gelatin 3.0 ng/ml, [2]SiO_2/Si-gelatin 15.0 ng/ml, [3]SiO_2/Si-gelatin 30.0 ng/ml, [4]SiO_2/Si-OPN 3.0 ng/ml, [5]SiO_2/Si-OPN 15.0 ng/ml, [6]SiO_2/Si-OPN 30.0 ng/ml

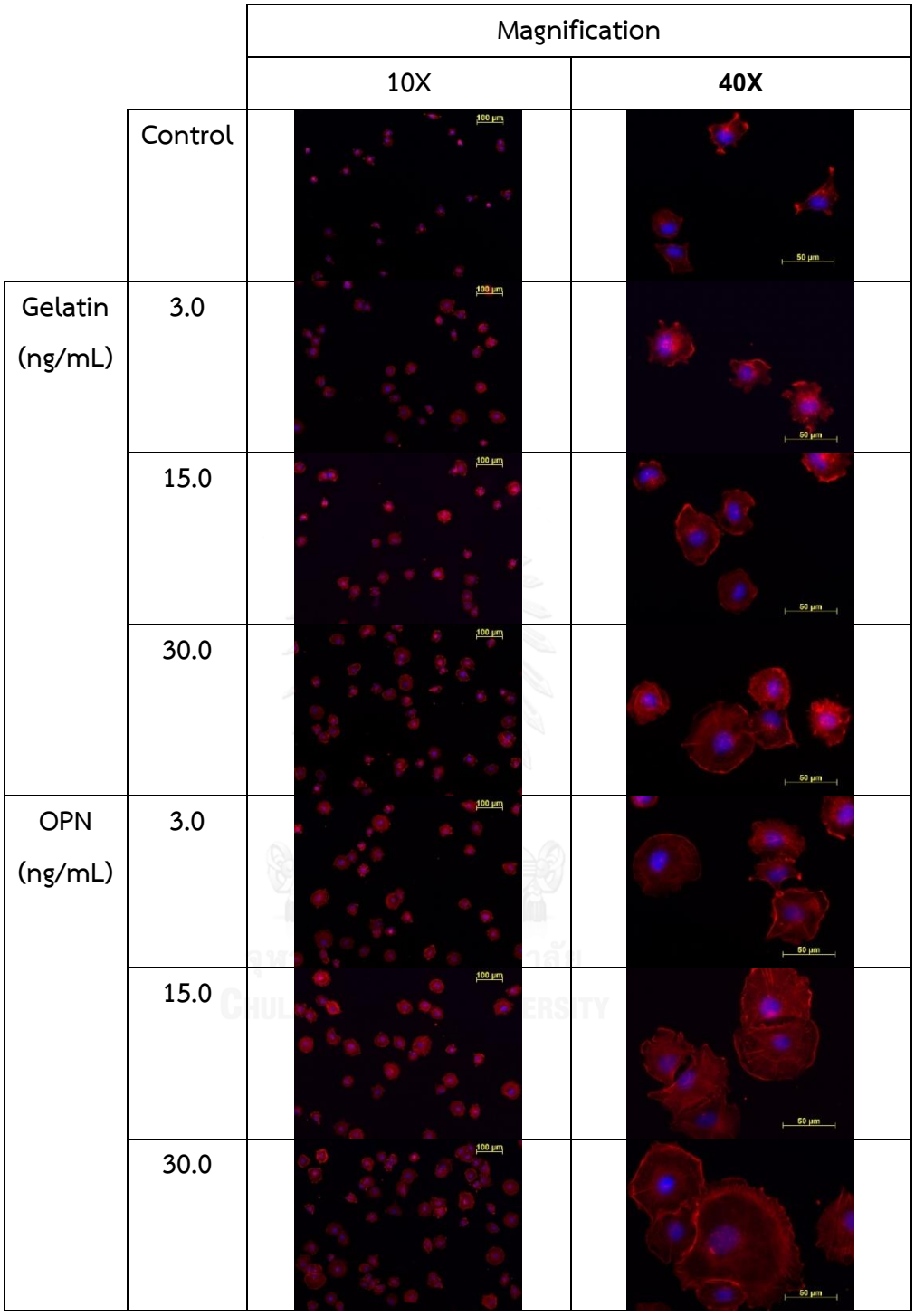

Figure 3.9 Micrographs of 10X and 40X magnification of MC-3T3-E1 cells cultured on surface for 2 hours of SiO_2 (control), SiO_2/Si-gelatin 3.0, 15.0 and 30.0 ng/ml (Gelatin 3.0, 15.0 and 30.0 respectively) and SiO_2/Si-OPN 3.0, 15.0 and 30.0 ng/ml (OPN 3.0, 15.0 and 30.0 respectively)

3.7 Expression of osteogenic marker genes

Expression of osteogenic marker genes **(Figure 3.10)** was determined by qPCR. Collagen type I (Col-1) is a major organic component of bone matrix. Osterix (OSX) and Runt-related transcription factor 2 (Runx2) are the key osteogenic differentiation regulatory genes. MC-3T3-E1 cells were cultured in OM for 1 day on SiO_2/Si-OPN and SiO_2/Si-gelatin. All relative gene expression levels were normalized to pristine glasses used as control samples. Expression levels of the following genes, namely Col-1 and Runx2 of MC-3T3-E1 cells on the SiO_2/Si-OPN increased in a dose-dependent manner and higher than those on pristine glass and SiO_2/Si-gelatin substrates. However, OSX gene was expressed after induction of Runx2 [28] so that MC-3T3-E1 cells cultured on surface for 1 day were found to give low level of OSX expression. These results indicated that OPN immobilization on surface-grafted PAA brushes can well promote osteoblast differentiation.

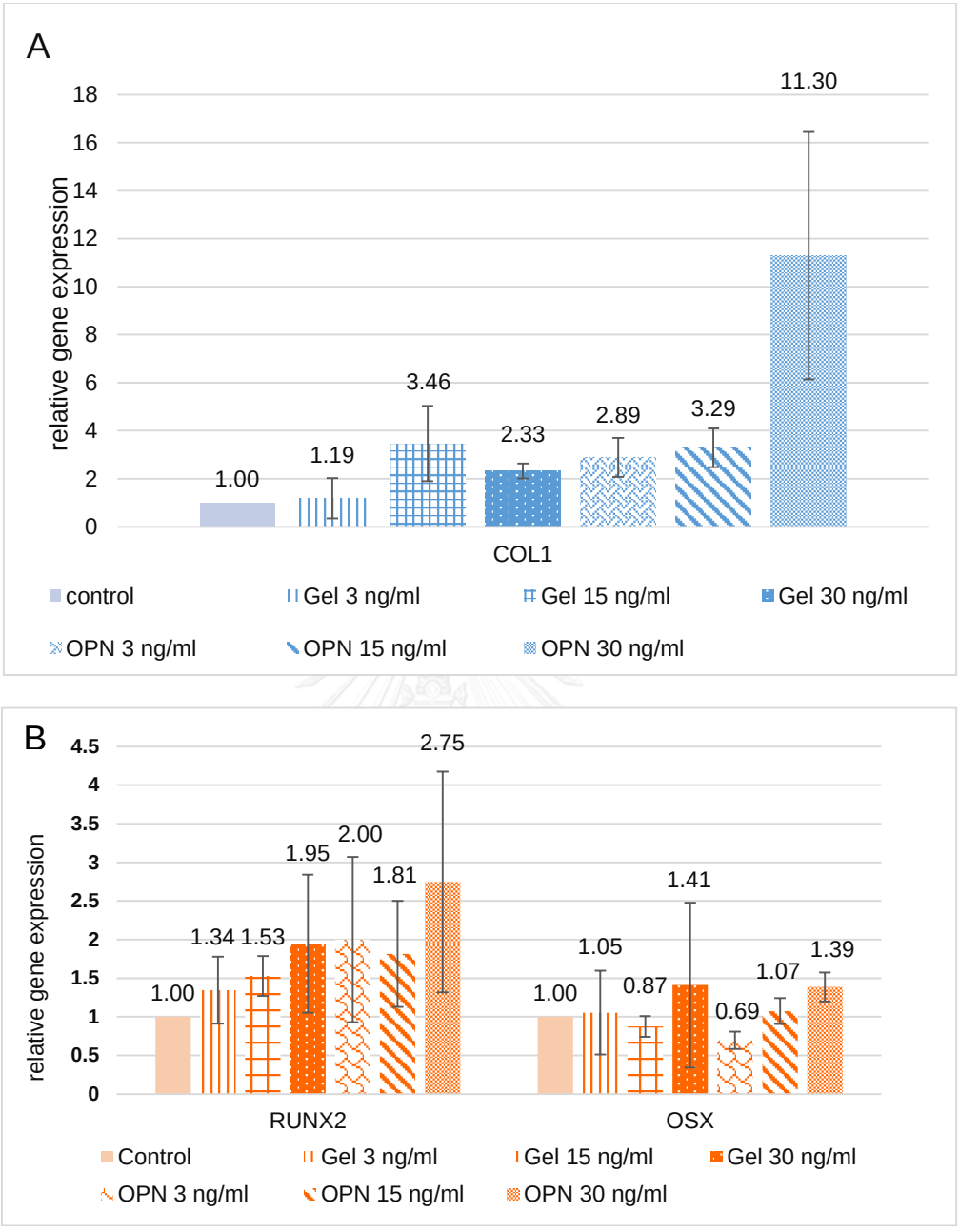

Figure 3.10 The relative gene expression level of gene markers (A) COL1 and (B) OSX and Runx2 in MC-3T3-E1 cells after cultured for 1 day on pristine glass (control), SiO_2/Si-gelatin (Gel) and SiO_2/Si-OPN (OPN)

3.8 Cell proliferation by MTT assay

MTT assay is a colorimetric assays for cell proliferation determination using yellow dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) which can be reduced by mitochondrial reductase present in metabolically active cells to providesthe violet formazan precipitates. Formazan can then be dissolved with organic solvent for measurement by spectrophotometry at 570 nm as shown in **Figure 3.11** [29]. The absorbance of the formazan solution increased with increasing the amount of living cells. MC-3T3-E1 cells were cultured on the SiO_2/Si-OPN for 2 hours, 1, 2, and 3 days before performing the assay, compared with cells cultured on unmodifed surface (control) and SiO_2/Si-gelatin. From **Figure 3.12**, it was found that at 2 hours, the amounts of living cells on SiO_2/Si-OPN and SiO_2/Si-gelatin were higher than the unmodified surfaces while there was no significant difference in cell viability on SiO_2/Si-OPN and SiO_2/Si-gelatin. At 1, 2 and 3 days, the increase of living cells compared with those cultured for 2 hours indicating that the cells were growing normally and cell viability on modified-surface remained higher than the unmodified surface. However, the cell viability on SiO_2/Si-OPN was still not significantly different from that of SiO_2/Si-gelatin. The cell proliferation ratio was calculated using **Equation 3.3** OD control and OD sample is defined as optical density of pristine glass surface and the sample, respectively.

$$\%\ cell\ viability\ = \frac{OD_{sample}}{OD_{control}} \times 100 \qquad \textbf{Equation 3.3}$$

At 1, 2 and 3 days, % cell viability of SiO_2/Si-OPN deceased slightly and was lower than that of SiO_2/Si-gelatin implied that OPN could not promote cell proliferation.

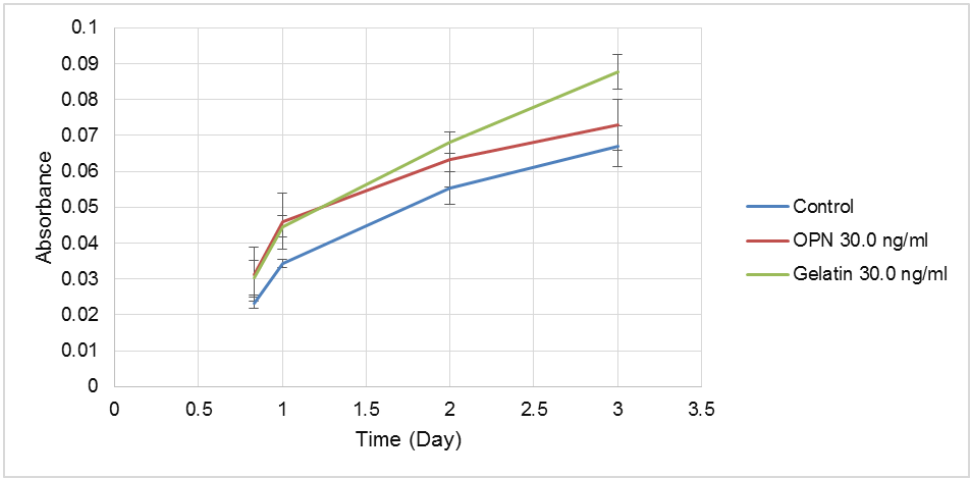

Figure 3.11 Cell proliferation determination at 2 hours, 1, 2 and 3 days using absorbance at 570 nm

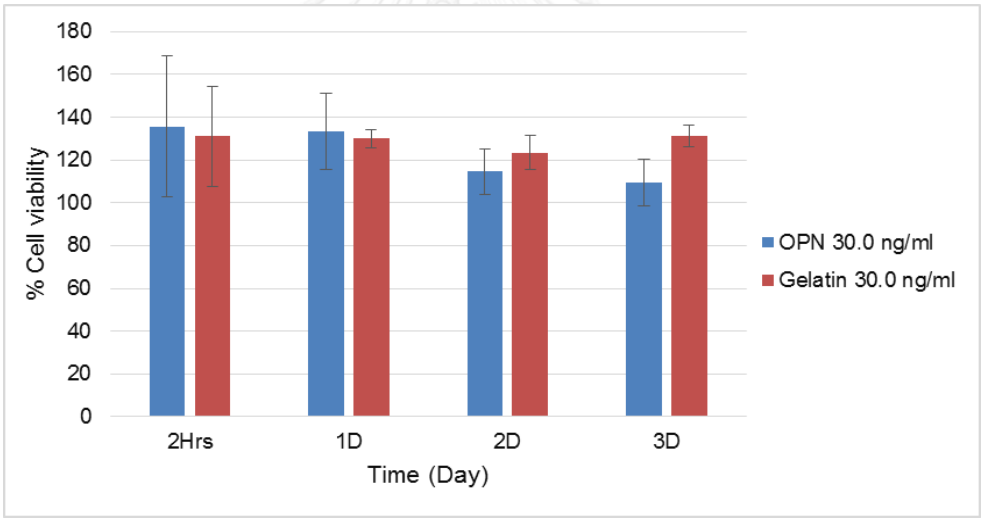

Figure 3.12 % Cell viability of OPN-modified surface and gelatin-modified surface at 2 hours, 1, 2 and 3 days

CHAPTER IV
CONCLUSION AND SUGGESTION

It has been successfully proven that PAA brushes can be grafted on glass substrates via surface-initiated RAFT polymerization. As determined by ^{1}H-NMR spectroscopy, the PAA formed in solution closely resembled that of theoretical molecular weight for the targeted DP of 100 suggesting that the RAFT polymerization was well-controlled.

Stepwise surface modification was verified by water contact angle measurements, Fourier transform-infrared spectroscopy (FT-IR) and x-ray photoelectron spectroscopy (XPS). The advancing water contact angle of 24.6 ± 7.8 indicated that the glass substrate became very hydrophilic once grafted with PAA. The characteristic C=O stretching peak appearing at 1723 cm^{-1} in the FT-IR spectrum of silica particles also confirmed the PAA brushes grafting. Simultaneous disappearance of such peak and the emergence of the characteristic C=O stretching at 1636 cm^{-1} strongly implied that amide bond was formed between carboxyl group of PAA and amino group of OPN after the step of OPN immobilization.. An increasing % nitrogen by XPS analysis can be used as an indication of the protein component of OPN once the surface was immobilized with OPN. Results from AFM analysis suggested that the silicon oxide surface was homogeneously and evenly covered with a layer of PAA brushes and OPN was effectively immobilized on the surface-grafted PAA brushes. As evaluated by Human OPN ELISA Kit, The percentages of OPN immobilization efficiency was higher than 95% at all concentration testes strongly suggesting that OPN can effectively be grafted on the surface-grafted PAA brushes. And the amount of surface-immobilized OPN per surface area can be tuned as a function of OPN concentration used in the immobilization step.

It was found that MC-3T3-E1 cells cultured on substrates immobilized with bioactive molecules (OPN and gelatin) spreaded better than those on pristine glass and SiO_2/Si-PAA substrates. Moreover, the degree of cell spreading on SiO_2/Si-OPN was apparently superior to that on SiO_2/Si-gelatin suggesting that OPN is more effective in

promoting bone cell adhesion than gelatin, a positive control. Expression of osteogenic marker of MC-3T3-E1 cells cultured on surface-modified glass substrates in OM for 1 day were determined by qPCR. . Expression levels of the following genes, namely Col-1, OSX and Runx2 of MC-3T3-E1 cells on the SiO_2/Si-OPN increased in a dose-dependent fashion and higher than those on pristine glass and SiO_2/Si-gelatin substrates. As determined by MTT assay, OPN could not promote cell proliferation. These results indicated that immobilized OPN on the surface-grafted PAA brushes could promote osteoblast adhesion and differentiation implying that this plant-derived OPN can potentially be used for bone tissue engineering applications in the future.

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APPENDIX

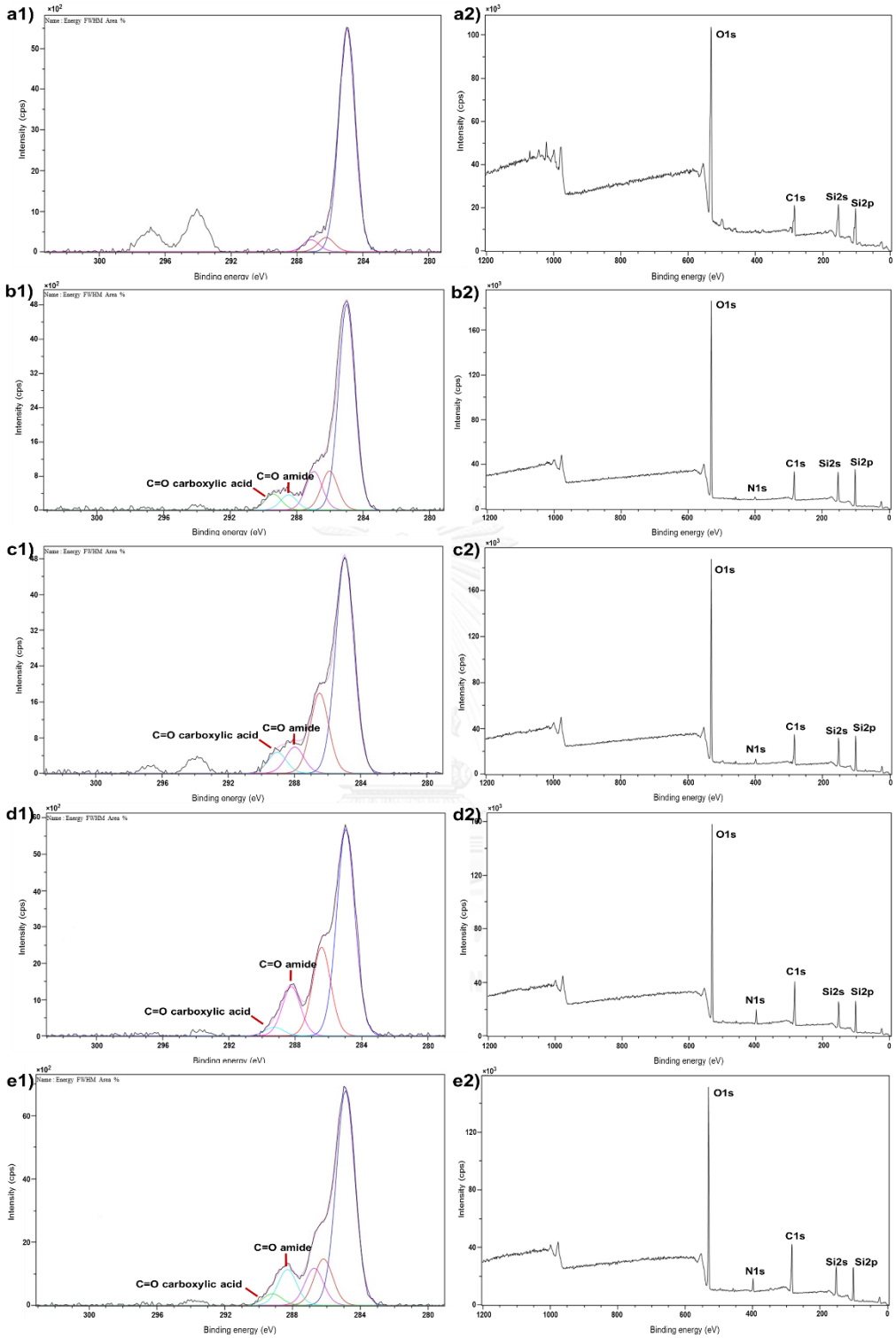

Figure A1 Deconvoluted and wide scan spectra of a.) pristine glass substrate cleaned by plasma cleaner b.) SiO_2/Si-PAA c.) SiO_2/Si-OPN 3 ng/mL d.) SiO_2/Si-OPN 15 ng/mL e.) SiO_2/Si-OPN 30 ng/mL

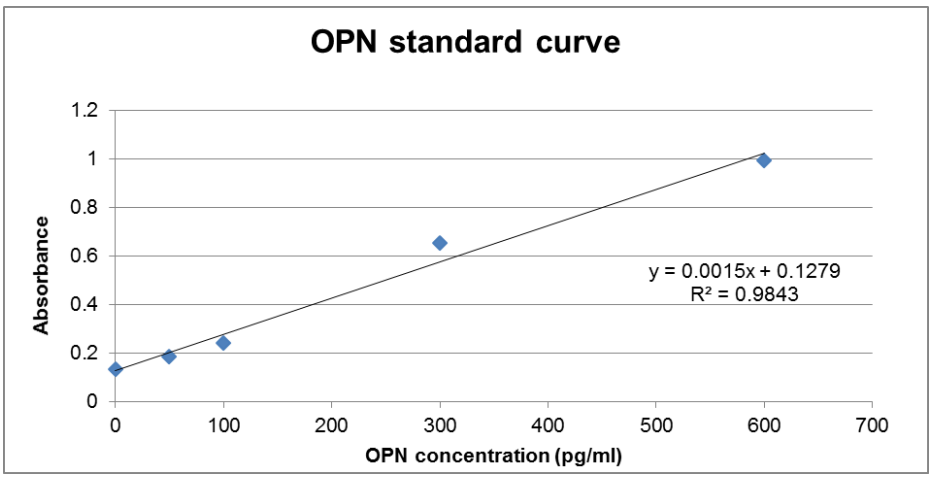

Figure A2 Standard curve for quantification of OPN immobilized on PAA by ELISA assay

Table A1 Absorbance measured by microplate reader at 450 nm for OPN concentration calculation

Batch	OPN concentration before immobilized on surface (ng/ml)	Absorbance
1	3.0	0.158
	15.0	0.169
	30.0	0.171
2	3.0	0.068*
	15.0	0.368*
	30.0	0.693*
3	3.0	0.096*
	15.0	0.483*
	30.0	0.644*

*Absorbance with 2-fold dilution of OPN solution after immobilization

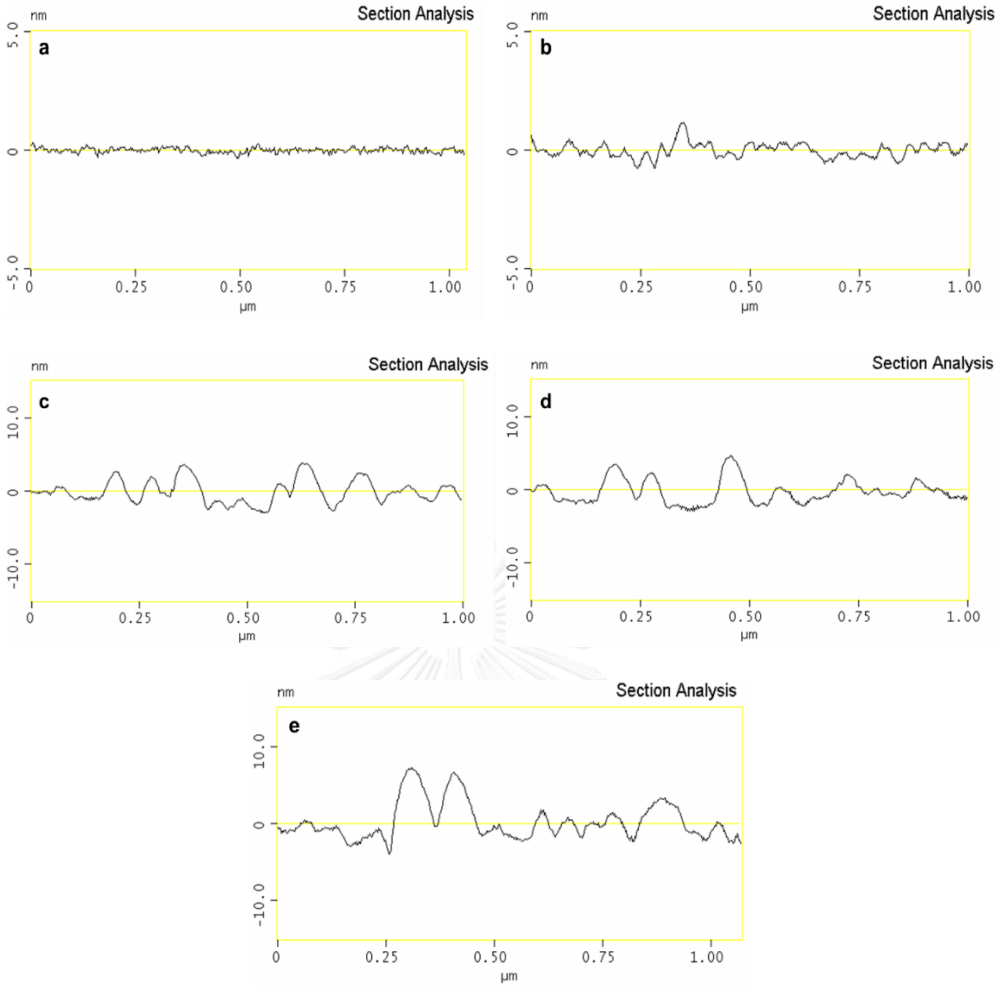

Figure A3 Section analysis of a.) SiO_2, b.) SiO_2/Si-PAA, c.) OPN 3.0, d.) OPN 15.0, e.) OPN 30.0 by AFM analysis

VITA

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