OPN Immobilization on Surface-grafted Poly(acrylic acid) Brushes to Promote Osteoblast Adhesion and Proliferation

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ABSTRACT

In this research, glass substrates were grafted with poly(acrylic acid) (PAA) brushes via surface-initiated reversible addition-fragmentation chain transfer (RAFT) polymerization. Osteopontin (OPN) synthesized from Tobacco plant was then immobilized on the surface-grafted PAA brushes using EDC/NHS as coupling agents yielding SiO$_2$/Si-OPN. Stepwise surface modification was verified by water contact angle measurements, Fourier transform-infrared spectroscopy (FT-IR) and x-ray photoelectron spectroscopy (XPS). MC-3T3-E1 cells cultured on the SiO$_2$/Si-OPN were better spreading than those on pristine glass substrates. Moreover, polymerase chain reaction (PCR) analysis indicated that expression levels of the following genes, namely collagen I (Coll I), osterix (Osx) and Runt-related transcription factor 2 (Runx 2) of MC-3T3-E1 cells on the SiO$_2$/Si-OPN were higher as compared with those on pristine glass substrates and the glass substrates immobilized with gelatin. 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.

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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 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 [1]. One of the ways for developing the implant surface is to immobilize biomolecules or proteins to increase of the implant efficiency. Osteopontin (OPN) is a protein which can promote endothelial cell adhesion to the environment and angiogenesis [2] 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 [3]. In this research, we used the glass substrate as a model of material to immobilize OPN for testing cellular responses. The glass substrates were first grafted with poly(acrylic acid)(PAA) to provide multiple active sites of carboxyl groups for OPN immobilization. Moreover, OPN synthesized from Tobacco, which was N-
glycosylated to mimic N-glycosylated proteins in the human body [4], was particularly 
chosen. Because this plant-derived OPN can be economically produced in large scale, it 
holds high commercial value if to be incorporated in medical products in the future. It is 
anticipated that the results from this research can be used as a guideline to modify the 
surface of other materials such as dental implant, guided tissue regeneration (GTR) 
membrane or scaffold in order to develop biomedical materials for promoting osteoblast 
adhesion and differentiation.

EXPERIMENTAL

A. Materials
3-Aminopropyltriethoxysilane (APTES), 4-dimethylaminopyridine (DMAP), 
dicyclohexylcarbodiimide (DCC), 4,4′-azobis(4-cyanovaleric acid) (ACVA), 4-cyano-4-
(phenylcarbonothioylthio)pentanoic acid (Chain transfer agent or CTA) and N-
hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich. Acrylic acid (AA) 
supplied by Aldrich was purified by vacuum distillation. N-(3-dimethylaminopropyl)-N′-
ethylenediamine hydrochloride (EDC) was purchased from TCL chemicals. Glass 
coverslips (i.d.=1.8 mm) were supplied by S.E. SUPPLY LTD PART. OPN extract was 
supplied by Department of Pharmacognosy and Pharmaceutical Botany, Faculty of 
Pharmaceutical Science, Chulalongkorn University.

B. Preparation of glass substrates immobilized with OPN (SiO$_2$/Si-OPN)
1. Silanization of glass substrates
Glass slides (diameter 18 mm.) were cleaned with a Plasma Cleaner for 5 minutes on both 
sides. Glass slides 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.

2. Preparation of glass substrates grafted with PAA brushes(SiO$_2$/Si-PAA)
ACVA (1 mmol, 0.21 g.) as initiator, DCC (1 mmol, 0.19 g.) and DMAP (0.1 mmol, 9.19 
mg) 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). Surface-initiated RAFT polymerization of AA was performed by firstly 
introducing a solution of CTA (0.2 mmol, 0.056 g), ACVA (0.05 mmol, 0.014 g) 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.

3. OPN immobilization on glass surface
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 were 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.

![Chemical structure](image)

**Scheme 1** Preparation of PAA-grafted substrates and subsequent OPN immobilization

### C. Cell culture and responses

1. **Determination of MC-3T3-E1 cell line morphology**

MC-3T3-E1 cells were cultured on glass substrates before and after OPN immobilization for 3 hours at 50,000 cells/well in 12-well plates. Then, the cells were fixed with 500 µL/well of 3.7% formaldehyde for 15 minutes and then added 0.1% triton-X-100 for 3 minutes to permeabilize cell membrane. After cell fixation, the cells were incubated in 1% BSA for reducing nonspecific background. The fixed cells were sequentially incubated with Rhodamine and DAPI for 20 minutes each to stain actin and nucleus, respectively. Cell morphology was observed by a ZEISS Observer.Z1 fluorescent camera.

2. **Gene expression analysis by real time-quantitative polymerase chain reaction (qPCR)**

MC-3T3-E1 cells were cultured for 1 day at 300,000 cells/well in 12-well plates. 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 converted 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. GAPDH forward sequencing primer (5’–3’) is CAC TGC CAA CGT GTC AGT GGT G and reverse sequencing primer (5’–3’) is GTA GCC CAG GAT GCC CTT GAG.

### RESULTS AND DISCUSSION

A. **Preparation and characterization of PAA-grafted substrates and subsequent OPN immobilization**

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 measurement shown in Table 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. The water contact angles went up after OPN immobilization suggesting that the surface was less hydrophilic. Chemical functionality was verified by FT-IR analysis of surface-functionalized silica particles undergoing similar chemical modification as the glass substrates. As can be seen in Figure 1, 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 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 1, an increasing % nitrogen from 1.01 of SiO$_2$/Si-PAA to 3.85% of SiO$_2$/Si-OPN can be used as an indication of the protein component of OPN on the SiO$_2$/Si-OPN

**Table 1** Water contact angle data and % element composition determined by XPS of surface-modified glass substrates

<table>
<thead>
<tr>
<th>Sample</th>
<th>Water contact angle ($^\circ$)</th>
<th>% element composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\theta_{\text{advancing}}$</td>
<td>$\theta_{\text{receding}}$</td>
</tr>
<tr>
<td>SiO$_2$</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>SiO$_2$/Si-ACVA</td>
<td>66.7 ± 1.5</td>
<td>35.0 ± 4.5</td>
</tr>
<tr>
<td>SiO$_2$/Si-OPN</td>
<td>44.7 ± 4.2</td>
<td>9.3 ± 6.8</td>
</tr>
</tbody>
</table>
| N/A – not measurable

**Figure 1** FT-IR spectra of a.) SiO$_2$, b.) SiO$_2$/Si-ACVA, c.) SiO$_2$/Si-PAA d.) SiO$_2$/Si-OPN. **B. Cell morphology of MC-3T3-E1 cells on SiO$_2$/Si-OPN**
MC-3T3-E1 cells were cultured in osteogenic media (OM) for 3 hours on SiO$_2$/Si-OPN having varied concentration (3, 15, 30 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). 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 2) showed 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.

C. Gene expression of MC-3T3-E1 cells line by qPCR

Expression of osteogenic marker genes (Figure 3) were determined by qPCR. Collagen type I (Col-1) is a major organic component of bone matrix. Osterix (OSX) and Runx-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$_2$/SiO$_2$-gelatin. All relative gene expression levels were normalized to pristine glasses used as control samples. 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$_2$/SiO$_2$-gelatin substrates. These results indicated that OPN immobilization on surface-grafted PAA brushes can well promote osteoblast differentiation.

![Figure 2](image_url)

**Figure 2** Micrographs of MC-3T3-E1 cell line cultured for 3 hours on a.) SiO$_2$, b.) SiO$_2$/Si-gelatin 3 ng/mL, c.) SiO$_2$/Si-gelatin 15 ng/mL, d.) SiO$_2$/Si-gelatin 30 ng/mL, e.) SiO$_2$/Si-PAA, f.) SiO$_2$/Si-OPN 3 ng/mL, g.) SiO$_2$/Si-OPN 15 ng/mL, h) SiO$_2$/Si-OPN 30 ng/mL.
Figure 3 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).

CONCLUSIONS

In summary, OPN were successfully immobilized on SiO$_2$/Si-PAA substrates that were prepared by surface-initiated RAFT polymerization of AA. The immobilized OPN can obviously promoted cell adhesion. Moreover, gene expression levels of OPN immobilized on surface were found to increase in a dose-dependent manner. These results indicated that OPN immobilization on the surface-grafted poly(acrylic acid) brushes could promote osteoblast adhesion and differentiation implying that OPN can potentially be used for bone tissue engineering applications.

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