Silane treatment of coated carbonate apatite scaffold affects bioactivity and cell viability

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Abstract. The aim of this study is to determine effects of surface treatment on coated-carbonate apatite (CO₃Ap) scaffold using silane coupling agent of 3-(trimethoxysilyl) propyl methacrylate. The effect of coating properties with and without surface treatment were investigated through in vitro bioactivity and biocompatibility. Firstly, CO₃Ap scaffold was fabricated by transforming ß-TCP scaffold via hydrothermal treatment. CO₃Ap scaffolds were treated in 2% silane solution before coating in chitosan solution (0.5%, 1% and 2%) by dipping method. The coated scaffold without silane treatment denoted as UTR while with silane treatment denoted as TR. It was found that, the presence of Si-OH group enhanced apatite growth on the coated scaffold. TR0.5 has capability to induce apatite growth for 7 days immersion in HBSS compared to UTR0.5. Rougher surface and different surface charge properties have influenced cell viability on the coated CO₃Ap scaffold. To be concluded, it is important to note that bioactivity and cell activity were forced by surface energy or topography, composition and electrostatic interaction.

1. Introduction

In developing bone scaffold, various forms of hydroxyapatite (HA) have been used in orthopaedic, dental or maxillofacial surgery. HA shows excellent biocompatibility and good osteoconductivity, however, the key drawback of HA is its stability in the bone as foreign substance. In other words, HA is difficult to be resorbed in the bone defect. The apatite found in human bone is not stoichiometric of Ca₁₀(PO₄)₆(OH)₂ but consisted by other ions mainly carbonate (CO₃²⁻) content and traces of Na⁺, Mg²⁺Fe²⁺, Cl⁻ and F⁻ [1]. Although the presence of these ions is low, they play an important role in the biochemical reactions of bone metabolism. As a result, CO₃²⁻ containing HA has gained much attention due to its chemical composition being closer to bone mineral and favourable for bone growth.

Brittleness of ceramic scaffold can be strengthen using polymer as coating material. Even though the strength will improve, the necessity of biodegradable polymers is also important to be
absorbed in host tissue. However, the concern in polymer-coated scaffold fabrication is the interfacial adhesion between bioceramics (inorganic phase) and polymer phase (organic phase). The interfacial bond strength within these dissimilar materials totally depends on the mechanical interlock. Due to chemical bonding does not exist in most bioceramics scaffold, surface treatment using silane coupling agents are often used for providing a strong chemical link in coating system.

Silane is the most commonly used due to its reactivity and ability to yield good and durable adhesion between the relevant substrates [2]. In this regard, the most popular silane coupling agent for bone reconstructions is the 3-(Trimethoxysilyl) propyl methacrylate (also called 3-methacryloyloxy propyl trimethoxysilane) (MPS). Its organofunctional group matches the chemical structure and reactivity of the inorganic/organic systems [3]. Silane coupling agents are often used for ceramic scaffolds to provide a strong chemical link between the oxide groups on the scaffold surface and the polymer molecules on the coating layer [4]. However, limited studies have been conducted to investigate the performance of coated carbonate-substituted apatite scaffold. Most previous researches focused on the silane treatment on composite-based bioceramic [4]. Therefore, this study aimed to investigate the effects of silane treatment on chitosan coated CO\textsubscript{3}Ap scaffolds. Bioactivity and cell behaviours on the coated CO\textsubscript{3}Ap scaffold with and without silanisation were investigated.

2. Materials and method

2.1. Materials

\(\beta\)-TCP, \(\text{Ca}_3(\text{PO}_4)_2\), powder with grade purum p.a, >96.0% was used as a main component to prepare \(\beta\)-TCP scaffold. Polyethylene-imine (PEI, 50 w/v%) was used as a dispersant in \(\beta\)-TCP slurry. Sodium dodecyl sulphate (SDS) powder was used as a pore forming agent. PVA acts as a binder in the ceramic slurry. These materials were purchased from Sigma Aldrich. Polyethylene glycol (PEG) in liquid form was purchased from Merck (Germany). PEG was used as a plasticizer. Denacol was supplied by Nagase Chemtex, Japan. Denacol was used as a gelling agent and crosslinker in \(\beta\)-TCP slurry. For transforming \(\beta\)-TCP scaffold to CO\textsubscript{3}Ap scaffold, disodium carbonate (\(\text{Na}_2\text{CO}_3\)) solution was used as a carbonate (CO\textsubscript{3}\textsuperscript{2-}) source. Medium molecular weight of chitosan (CS, 75-85% deacetylation) was used as a natural polymer solution for coating purpose. CS was purchased from Sigma Aldrich. 3-(Trimethoxysilyl) propyl methacrylate (MPS)-98% or silane A174 is a silane coupling agent for the surface treatment. Silane was purchased from Sigma Aldrich. Methanol, 99.9% was purchased from JT Baker. Methanol was used as a silane solvent. Acetic acid (100% anhydrous) was used as a CS solvent while glutaraldehyde, GA, 50% solution in water was used as a crosslinker to bind amine group in CS.

2.2. Methods

2.2.1 Fabrication of CO\textsubscript{3}Ap scaffold. \(\beta\)-TCP scaffold was fabricated using gelate-freeze casting method. \(\beta\)-TCP slurry was prepared by mixing 60 wt.% of \(\beta\)-TCP powder and 40 wt.% distilled water based solution. Firstly, 4 wt.% of PVA was dissolved in the distilled water. After that, PEG was mixed in the PVA solution. \(\beta\)-TCP powder was added in the mixture as ceramic slurries. The slurries were homogeneously mixed by using mechanical stirrer at 600 rpm. Then the slurry was added with PEI and continuously stirred. After that, SDS was added under 900 rpm stirring until the slurry 1.5 times expanded. Denacol was lastly added to maintain the slurry expansion. The casting process of the slurry was done by pouring ceramic slurries into mold. The slurry was dried at room temperature for 1 h to complete the gelling phase. Then, the molds were transferred into -30 °C freezer for 3 h. After that, the molds were placed in freeze drier using Freeze Dry System (Labconco) for 24 h. The green bodies were sintered in an air furnace at a temperature of 1115 °C. Fabrication of CO\textsubscript{3}Ap scaffold was done using hydrothermal treatment method. \(\beta\)-TCP scaffold was used as precursors. The precursors were soaked in 5 mol/L of Na\textsubscript{2}CO\textsubscript{3} solution in hydrothermal vessel. The vessels were kept at 200 °C in oven for 5 days.
After that, the fabricated CO3Ap scaffolds were soaked in boiling distilled water to wash the excessive sodium in the scaffold. Then, the scaffolds were dried at 80 °C for 1 h.

### 2.2.2 Chitosan-coated CO3Ap scaffold without and with surface treatment

The silanisation process was performed by soaking the CO3Ap scaffolds in the silane solution for 120 min. The silane solution was prepared by depositions from an aqueous methanol solution. This process included a 95% methanol/5% water solution adjusted to a pH 4.5–5.5 with acetic acid. The silane was added while stirring to yield a final concentration of 3%. After soaking for 120 min, the scaffolds were heat-treated in the oven at 160ºC for 2 h. In this study, this is denoted as silanised-CO3Ap scaffolds. The silanised-CO3Ap scaffolds were cross-linked with 2% (v/v) GA in ultrapure water overnight with slow agitation. Each sample was rinsed three times with deionized water, then heated and stored for the coating process.

CS solutions were prepared by dissolving the powder in distilled water (250 mL) to produce three different concentrations (0.5%, 1%, and 2%) of chitosan, with 2% (v/v) acetic acid. The solution was stirred to dissolve the chitosan completely. Silanised-CO3Ap scaffolds were then soaked into the chitosan solution. Vacuum was then used for 4 h to force the CS solution to infiltrate the porous structure of the CO3Ap scaffolds. Scaffolds were dried in a vacuum oven at 80ºC for 24 h. This coated-CO3Ap scaffolds has been denoted as treated scaffold. The same coating procedures were carried out for CO3Ap scaffolds without the silane treatment, which is denoted as untreated. The composition and designation of the coatings with the respective scaffolds are summarized in Table 1.

| Sample designation | Silane treatment | Chitosan (CS) concentration (w/v%) |
|--------------------|-----------------|-----------------------------------|
| Neat CO3Ap         | -               | -                                 |
| Silanised- CO3Ap   | √               | -                                 |
| UTR0.5             | -               | 0.5                               |
| TR0.5              | √               | 0.5                               |
| UTR1               | -               | 1                                 |
| TR1                | √               | 1                                 |
| UTR2               | -               | 2                                 |
| TR2                | √               | 2                                 |

### 2.3 Characterisations

Hanks’ balanced salt solution (HBSS, Gibco), was used as a supporting solution for the CO3Ap scaffolds in an in vitro bioactivity test. The cylinder-shaped of scaffolds were immersed in 25 mL of HBSS to elicit a mineralisation reaction. After soaking, the scaffolds were taken out and rinsed with distilled water to remove residual HBSS and then immediately dried at 50 °C for 24 h. The morphology and composition of the mineral layers formed on the scaffolds were characterised by energy dispersive X-ray spectroscopy (EDX, EDAX Genesis). Cell viability using human osteoblast cell (hFOB). The viability assay was carried out using PrestoBlue™ Cell Viability Reagent using an automated microplate reader according to standard protocol provided by the manufacturer after 1, 2, 3, 5 and 7 d of culture.

### 3. Results and discussions

#### 3.1 Bioactivity

The capability of UTR and TR scaffolds to induce and form bonelike apatite growth were determined by the changes of Ca/P ratio as presented in Table 2. For 7 days immersion, the Ca/P of the TR scaffolds have higher ratio than those of UTR scaffolds. It revealed a Ca-rich amorphous calcium phosphate
(ACP) with Ca/P ratio of 1.98, 1.67 and 1.58 on TR0.5, TR1 and TR2 respectively. The formation of the Ca-rich ACP was assisted by functional group of Si–OH. The Ca-rich ACP was assumed to take place in consecutive accumulation of the calcium ions, which makes positive charge increased. The Ca-rich ACP on the TR scaffold therefore interacts specifically with the negative phosphate ions in the fluid to form a Ca-deficient (poor) ACP. This type of Ca-deficient (poor) ACP was observed as a precursor, which eventually crystallizes into bonelike apatite that lowered the Ca/P ratio of the TR scaffolds after 28 days immersion, i.e; 1.65, 1.64 and 1.55 for TR0.5, TR1 and TR2, respectively.

Table 2. The molar Ca/P ratio of UTR and TR scaffolds after 7 and 28 days immersion in HBSS solution

| Scaffold | Ca/P ratio |
|----------|------------|
|          | 0          | 7          | 28         |
| Neat CO₃Ap | 1.66      | 1.78       | 1.58       |
| UTR0.5    | -          | 1.85       | 1.86       |
| TR0.5     | -          | 1.98       | 1.65       |
| UTR1      | -          | 1.65       | 1.68       |
| TR1       | -          | 1.67       | 1.64       |
| UTR2      | -          | 1.36       | 1.68       |
| TR2       | -          | 1.58       | 1.55       |

The mechanism of apatite formation starts when the TR scaffold is immersed in the HBSS, Ca²⁺ in the scaffold exchange with H⁺ in the SBF solution. This leads to the formation of silanol (-Si–OH) in the surface layer at the scaffold–HBSS, and eventually the production of a negatively charged surface with the functional group (-Si–O⁻). The Ca²⁺ in the HBSS solution are first attracted to the interface between coating and solution to form Ca-rich ACP. Consequently, the ionic activity product of the apatite in the interface is high enough to precipitate apatite on the TR surface [5]. Thus, the results suggest that the changes of apatite-forming behaviour were affected by the surface compositional of CO₃Ap scaffold. The bioactive materials with a superior in vitro apatite-forming ability determine the directly bonding to living bone in vivo[6].

3.2. Relation between surface charge and cell proliferation

Both UTR0.5 and TR0.5 scaffolds were used to be further characterized using zeta potential and cell study. Figure 1 shows the zeta potentials for all CO₃Ap scaffolds at pH 7 in HBSS. All scaffolds are negatively charged, and the absolute zeta-potential comparison sequence was indicated as, silanised-CO₃Ap < neat CO₃Ap < UTR0.5 < TR0.5. Considering CO₃Ap scaffold, the surface was found to be negatively charged represented from the presence of OH- group. After silanisation, CO₃Ap became less negatively charged, as could be expected from the presence of silanol groups. This was related to the nonpolar group of CH₃⁺. Zeta potential of coated CO₃Ap were portrayed more negatively charged i.e; -14 mV (TR0.5) and -12.95 mV (UTR0.5) than those of neat and silanised-CO₃Ap scaffolds. It is known that, CS possesses a higher positive surface charges due to more extensive protonation of its amino groups at pH below its pKₐ,~6.5. However, this explanation is disputable whereby HBSS at pH 7 used in the present study caused the decrement of zeta potential upon neutralization of the respective solution used. This finding is in agreement with results reported by Loh et al. [7]. They studied that CS exhibited negative charges at pH 7.4 and positive charges at pH 6.0 as the zeta potentials measured in HBSS. In addition, higher negatively charge of TR0.5 might be resulted from well-dispersed of CS coating on the TR0.5 surface due to chemical link bonding compared to UTR0.5
Figure 1. Zeta potential of different surface CO$_3$Ap scaffolds. * indicates p < 0.05 is significantly different.

Figure 2 shows cell proliferation of hFOB cells on the neat CO$_3$Ap, silanised-CO$_3$Ap, UTR0.5 and TR0.5 scaffolds. Cells seeded on a tissue culture plate without scaffold were taken as a control. The silanised-, UTR and TR CO$_3$Ap scaffolds showed an increase of cell proliferation than control. The silanised-CO$_3$Ap scaffold showed an increase in cell proliferation compared with others all the time. Rougher surface by silanised-CO$_3$Ap scaffold (as indicated in Figure 3) is believed has provided higher adhesion of hFOB cells. The rougher surface increased the surface energy which resulted in better chemical bonding with cells.

Figure 2. The results of PrestoBlue assay for silanised-, UTR0.5 and TR0.5 CO$_3$Ap scaffolds in the presence of hFOB cells for 7 days incubation.

Figure 3. SEM images and EDX spectra shows elemental composition of (a) neat CO$_3$Ap and (b) silanised-CO$_3$Ap scaffolds.

As indicated, the silanised-CO$_3$Ap scaffold has less negative charge but the proliferation was superior among others. According to Qin et al. [8], osteoblast cells tended to adhere and proliferated more when the magnitude of negative zeta potential increased. Therefore, in this study cells were able...
to proliferate more on silanised-CO$_3$Ap surface with a less negative zeta potential (-9.885 mV) could be related with rough surface of silanised-CO$_3$Ap, which provided adhesion promotion with more micromechanical retention. In addition, the silane treatment does not have intrinsic toxicity to be used for cell growth [9]. As already known, cell surface is negatively charged. Due to same negatively charged between the cell and the material no direct electrostatic interaction occurred. Thus, why cells can proliferate on negative surface charge could be explained as followed. Whenever materials encounter a biological environment, the protein adsorption process happened within a few seconds. The protein is amphoteric polyelectrolyte with both acidic and basic peptide. Therefore, it behaves as a bridge between positive or negative groups on cell surfaces or modified CO$_3$Ap surface. To be concluded, these results provided the outcomes of in vitro studies which can be manipulated to select the most optimal performance of surface functionality for in vivo studies.

4. Conclusion
The functional group of Si-OH by treated CO$_3$Ap scaffolds induced the precipitation bonelike apatite in HBSS. Rougher the scaffold surface exhibited by silane treatment has increased cell viability. Utilization of silane treatment on the coated scaffold provided mechanism of enhancement in bioactivity and cell growth for successful bone regeneration.

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