

# Mesoporous Silica-Coated $\beta$ -TCP Granules Prepared Using Alginate and *In vitro* Evaluation

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## To cite this article:

Atsumasa Shishido, Yudai Shigarami, Erath Beeran Ansar, Hari Krishna Varma, Yoshiyuki Yokogawa. Mesoporous Silica-Coated  $\beta$ -TCP Granules Prepared Using Alginate and *In vitro* Evaluation. *International Journal of Biomedical Materials Research*.

Vol. 8, No. 1, 2020, pp. 8-13. doi: 10.11648/j.ijbmr.20200801.12

Received: June 9, 2020; Accepted: June 23, 2020; Published: July 13, 2020

**Abstract:**  $\beta$ -Tricalcium phosphate ( $\beta$ -TCP) is widely used, along with bone-derived growth factor, due to its poor osteogenic ability. Mesoporous silica (MPS)-coated  $\beta$ -tricalcium phosphate ( $\beta$ -TCP) granules were prepared to improve protein loading capability, and *in vitro* evaluations of this material were carried out.  $\beta$ -TCP powder containing 2 wt%  $\text{Al}_2\text{O}_3$  and 6 wt%  $\text{SiO}_2$  was prepared *via* mechanochemical synthesis. A sodium alginate solution containing  $\beta$ -TCP powder was transferred into a calcium chloride solution, and the obtained spherical beads were heated at 1100 to 1300°C to produce TCP granules. The X-ray diffraction (XRD) profile of the  $\beta$ -TCP granules containing 2 wt%  $\text{Al}_2\text{O}_3$  and 6 wt%  $\text{SiO}_2$  was identical to that of the single  $\beta$ -TCP phase when heated to 1300°C. The compressive strengths of the TCP granules prepared using alginate were remarkably improved compared with those of TCP granules prepared using a pan-type granulator. A silica interlayer was formed on the  $\beta$ -TCP granules containing 2 wt%  $\text{Al}_2\text{O}_3$  and 6 wt%  $\text{SiO}_2$  via magnetron sputtering prior to the coating of the MPS. MPS coatings on  $\beta$ -TCP granules containing 2 wt%  $\text{Al}_2\text{O}_3$  and 6 wt%  $\text{SiO}_2$  was carried out by a dip-coating method after silica interlayer coatings, and the  $\beta$ -TCP granules containing 2 wt%  $\text{Al}_2\text{O}_3$  and 6 wt%  $\text{SiO}_2$  were covered by the MPS particles. A silica interlayer may offer bonding between the  $\beta$ -TCP granules and MPS coating. The Alamar blue assay of the MPS-coated TCP granules exhibited excellent cell viability as well as a high protein-adsorption capacity.

**Keywords:** Mesoporous Silica, Coatings,  $\beta$ -TCP, Compressive Strength, Cytocompatibility

## 1. Introduction

$\beta$ -Tricalcium phosphate ( $\text{Ca}_3(\text{PO}_4)_2$ ) and  $\beta$ -TCP) ceramic material is regarded as an ideal material for bone substitution in the body [1, 2], since the biodegradable characteristics of  $\beta$ -TCP enhance bone ingrowth into implants.  $\beta$ -TCP ceramic materials also have been used along with bone-derived growth factors for the regulation of bone formation, due to its poor osteogenic ability. Mesoporous silicate (MPS) materials have been extensively studied [3] because of their high loading capacities as protein carriers and their well-defined spaces for the enclosure of proteins [4].

We previously reported upon MPS coatings on hydroxyapatite (HAp) dense sintered body achieved by the dip-coating method [5] and by the vapor-phase coating method [6]. The quantity of protein adsorbed on the MPS-coated HAp dense sintered body was improved by the MPS coatings on HAp dense sintered body. However, the coating of MPS on HAp granules was not successful, while it was achieved when a silica layer was formed on the HAp granules prior to the MPS coating [7]. The use of a silica layer as an interlayer between HAp granules and MPS coatings may prevent reactions from occurring on the HAp granules, resulting in the formation of an MPS film by the hydrolysis

and condensation reaction of TEOS.

Sintered TCP ceramic materials exhibit relatively low bending strengths in comparison to hydroxyapatite ( $\text{Ca}_5(\text{PO}_4)_3(\text{OH})$ , HAp) ceramics since  $\beta$ -TCP transforms into  $\beta$ -TCP with lattice expansion at temperatures greater than  $1120^\circ\text{C}$  [8]. Many studies have reported upon the suppression of the phase transformation of  $\beta$ -TCP ceramics using additives such as  $\text{MgO}$ ,  $\text{SiO}_2$  et al. It has also been reported that additives of  $\text{SiO}_2$  did not improve the mechanical strength and bioactivity of  $\beta$ -TCP ceramics [9]. While, a high sintering strength can be achieved by using  $\text{SiO}_2$  along with  $\text{Al}_2\text{O}_3$  as additives [10]. We previously reported  $\beta$ -TCP granules with  $\text{Al}_2\text{O}_3$  and  $\text{SiO}_2$  prepared using a pan-type granulator. It was determined that the compressive strength of the  $\beta$ -TCP granules with  $\text{Al}_2\text{O}_3$  and  $\text{SiO}_2$  achieved by heating at  $1200^\circ\text{C}$  was  $4.16 \pm 1.88$  MPa (with an apparent porosity of 62.0%), which is comparable to that of commercial products [11]. MPS coatings on  $\beta$ -TCP granules prepared using a pan-type granulator were successfully achieved by forming a silica interlayer on the  $\beta$ -TCP granules prior to the MPS coatings [12].

The aim of this study was to investigate the further improvement of the mechanical strength and biocompatibility of MPS-coated  $\beta$ -TCP granules.  $\beta$ -TCP granules containing  $\text{Al}_2\text{O}_3$  and  $\text{SiO}_2$  were prepared using sodium alginate. A silica interlayer on the  $\beta$ -TCP granules was formed *via* reactive radiofrequency (rf) magnetron sputtering prior to the coating with MPS films. Compressive strength measurements and *in vitro* evaluations of the MPS-coated  $\beta$ -TCP granules were then carried out.

## 2. Experimental

### 2.1. MPS Coating on $\beta$ -TCP Granules

$\beta$ -TCP powder was prepared by a mechanochemical synthesis method as previously reported [13]. A mixture of calcium hydrogen phosphate dihydrate ( $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ ) and calcium carbonate ( $\text{CaCO}_3$ ) at a Ca/P ratio of 1.5 was placed in 90 wt% pure water to obtain a slurry. The slurry was ball-milled using zirconia balls in a pot mill at 110 rpm for 48 hours at room temperature. The reacted slurry was then dried at  $50^\circ\text{C}$  for 10 days and heat-treated in air at  $720^\circ\text{C}$  for 8 hours to produce  $\beta$ -TCP powder. The  $\beta$ -TCP powder was then prepared by adding 2 wt% of  $\text{SiO}_2$  and 6 wt% of  $\text{Al}_2\text{O}_3$  to obtain a slurry and subjected to the aforementioned process to produce a TCP powder containing  $\text{SiO}_2$  and  $\text{Al}_2\text{O}_3$ , which is referred to as TCP-AlSi powder.

A 1 wt% calcium chloride ( $\text{CaCl}_2$ ) aqueous solution was added dropwise to a 1 wt% solution of sodium alginate containing 20 wt% TCP or TCP-AlSi powder. The obtained granules were washed with Milli-Q water, dried, and heated at 1100 to  $1300^\circ\text{C}$  for 3 hours. The granules prepared using TCP are referred to as TCP-A granules, while those prepared with TCP-AlSi are referred to as TCP-AlSi-A granules. The granules were also prepared using a pan type granulator along with polyvinyl alcohol as binder. The pan type granulator

made granules using TCP or TCP-AlSi are referred to as TCP-AlSi-P granules.

A silica interlayer was formed on both the TCP-A or TCP-AlSi-A granules by the reactive-plasma sputtering method. Reactive plasma sputtering was performed using a rf magnetron sputtering apparatus. The gas pressure was 10 mTorr under Ar and 5%  $\text{O}_2$ , and the target material used was pure silicon. The sputtering process was carried out for 5 times, and the granules were turned over at each process to be completely covered by Si-O interlayer. The obtained granules are referred to as interlayer/TCP-AlSi-A granules.

The interlayer/TCP-AlSi-A granules were placed into a solution containing tetraethyl orthosilicate (TEOS), nitric acid and cetyltrimethylammonium bromide (CTAB) as a structure-directing agents (SDAs), and dried and heated at  $500^\circ\text{C}$  for 3 hours. The obtained MPS-coated interlayer/TCP-AlSi-A granules are referred to as MPS/interlayer/TCP-AlSi-A granules.

### 2.2. Characterization

The crystalline phases of the products were analyzed by X-ray powder diffraction (XRD) method using an X-ray diffractometer at 40 kV and 20 mA with  $\text{CoK}$   $\beta$  radiation. Identification of the phases was achieved by comparing the diffraction patterns with ICDD (JCPDS) standards. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) observations, as well as energy-dispersive X-ray (EDX) analysis of the products, were performed. SEM and TEM observations were performed by field-emission SEM (FE-SEM, JSM-6500FS, JEOL) at 15–20 kV and conventional TEM (H-7000, Hitachi, operated at 70 kV). To avoid charging problems during the SEM and TEM observations, osmium was deposited onto the product surface using an osmium plasma coater (OPC60A, Filgen).

The products were dried in a vacuum at  $150^\circ\text{C}$  for 24 hours to remove the adsorbed water, and the Brunauer–Emmett–Teller (BET) surface areas and Barrett–Joyner–Halenda (BJH) pore size distributions of the products were measured using an Autosorb-1 (Yuasa Ionics Inc., Japan).

The compressive strengths of the as-prepared MPS/interlayer/TCP-AlSi-A granules were measured as follows. The diameter of each specimen was measured using a micrometer (M300, Mitsutoyo, Japan), and each specimen was individually placed on the stage of a universal tester (AG-10, Shimadzu Co., Japan). The load at the breaking point was measured at a crosshead speed of 1.0 mm/min, and the compressive strengths of the round materials were calculated according to a previously reported equation [14]. The procedure was repeated 10 times for each specimen.

### 2.3. Cytocompatibility Test

The cytocompatibilities of the TCP and MPS/interlayer/TCP-AlSi-A granules were tested *via* Alamar Blue (AB) assay by assessing the cell viability and proliferation based on the reduction potential of the metabolically active cells. L929 cells were seeded in

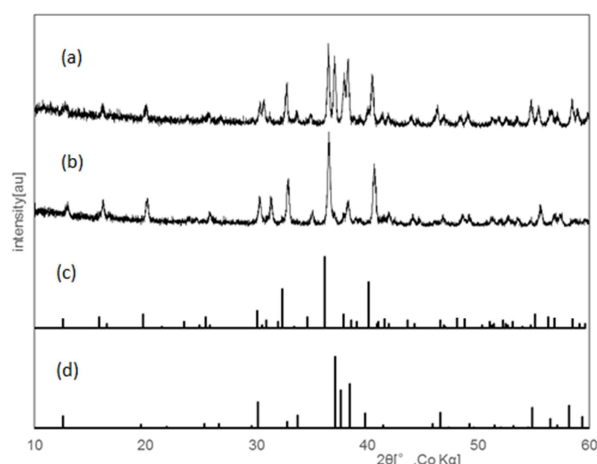
transparent 96-well plates and exposed to the materials (TCP and MPS/interlayer/TCP-AlSi-A granules) at concentrations of 0.1 mg and 0.3 mg for 24 hours. AB reagent was added to each well as per the protocol. The fluorescence of the cells was measured at 560 nm excitation and 590 nm emission wavelengths using a plate reader (Hidex Chameleon). Experiments were performed six times for each material concentration of 0.1 mg and 0.3 mg.

The cells were cultured for 24 hours on the samples, washed twice with Sorensen's phosphate buffer (SPB), and fixed with 1% glutaraldehyde in SPB for 24 hours. The samples were dehydrated in increasing concentrations of ethanol (30%, 50%, 70%, 90%, and 100%) and were critical-point-dried. Next, the samples were mounted on aluminum stumps and coated with gold, and the morphologies of the cells were assessed by environmental SEM (FEI Quanta 200).

### 3. Results and Discussion

#### 3.1. Materials

Figure 1 shows the XRD profiles of the TCP-AlSi-A granules that were prepared using alginate at Ca/P ratios of 1.5 and 1.45 and heated at 1100°C. The XRD profile of the TCP-AlSi-A granules prepared at a Ca/P ratio of 1.45 was identical to that of the  $\beta$ -TCP phase without any by-products such as aluminum oxide, silicon oxide and aluminum phosphate; however, that of the TCP-AlSi-A granules prepared at a Ca/P ratio of 1.5 exhibited additional peaks that correspond to the peaks of the HAp phase. TCP-AlSi powder was added to a calcium chloride solution to obtain TCP-AlSi-A granules; the additional calcium content may thus react with  $\beta$ -TCP, resulting in the formation of a HAp phase during heat treatment [10].



**Figure 1.** XRD profiles of the TCP-AlSi-A granules prepared at a Ca/P ratio of 1.5 (a) and the TCP-AlSi-A granules prepared at a Ca/P ratio of 1.45 (b) heated at 1100°C, XRD peak diagram derived from ICDD data of  $\beta$ -TCP (#9-169)(c) and HAp (#9-432) (d).

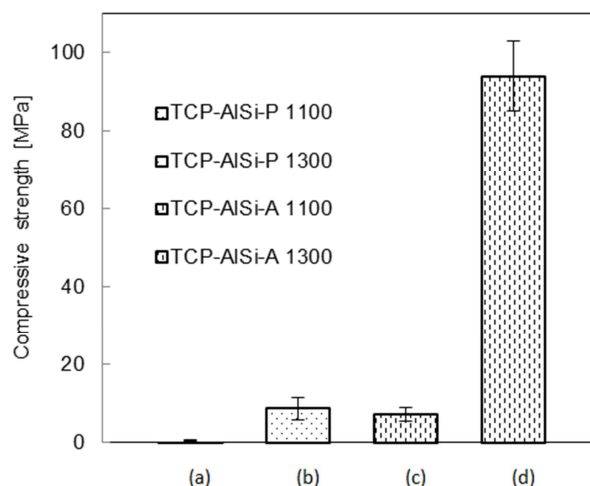
TCP-AlSi-A granules at a Ca/P ratio of 1.45 were heated up to at 1200 to 1300°C, and the obtained XRD profiles were also found to be consistent with that of  $\beta$ -TCP. The  $\text{SiO}_2$  and  $\text{Al}_2\text{O}_3$

additives may inhibit the phase transformation of  $\beta$ -TCP into  $\beta$ -TCP [10].

The peaks of the TCP-AlSi-A granules shift to higher angles, compared with those of XRD diagram derived from ICDD data, which may be due to the  $\text{SiO}_2$  and  $\text{Al}_2\text{O}_3$  solid-dissolved in TCP phase. Al may partly substitute for Ca, and the ionic radius of Al is smaller than that of Ca, which may decrease the unit cell size of TCP.

Figure 2 shows the compressive strength of the TCP-AlSi-P and TCP-AlSi-A granules, heated at 1100 °C and 1300 °C. Comparing the compressive strengths of the granules heated at 1100 °C, that of TCP-AlSi-A granules is  $7.20 \pm 1.71$  MPa and ten times higher than that of TCP-AlSi-P granules. The apparent porosity of the former is 29.5% and that of the latter is 76.4%. The TCP-AlSi-A granules were formed via hydrogels by adding sodium alginate, while the TCP-AlSi-P granules were prepared using pan type granulator with polyvinyl alcohol solution. Sodium alginate and polyvinyl alcohol as binder form agglomeration of powders, and the agglomeration degree of the former may be much higher than that of the latter.

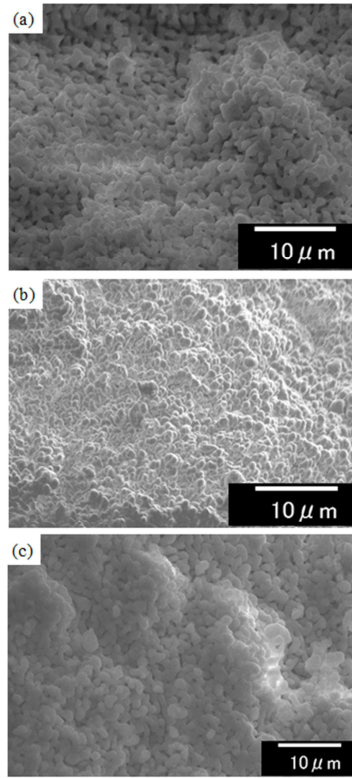
When the heating temperature was raised up to 1300 °C, the compressive strengths of TCP-AlSi-P granules became considerable high to be  $8.66 \pm 2.85$  MPa, as the additives,  $\text{Al}_2\text{O}_3$  and  $\text{SiO}_2$ , may suppressed the phase transformation from  $\beta$ -TCP to  $\beta$ -TCP and the high-temperature sintering process induced the high densification of the sintered body. That of TCP-AlSi-A granules remarkably improved, which may be due to the higher density of the granules.



**Figure 2.** Compressive strengths of TCP-AlSi-P granules heated at 1100 °C (a) and 1300 °C (b), as well as TCP-AlSi-A granules heated at 1100 °C (c) and 1300 °C (d).

Figure 3 shows the SEM images of the TCP-AlSi-A, the interlayer/TCP-AlSi-A and the MPS/interlayer/TCP-AlSi-A granules. Fine powders having 1-2  $\mu\text{m}$  size are linked together on the TCP-AlSi-A granules (Figure 3 (a)), heated at 1100°C. The round shaped particles are embedded on the granules (Figure 3 (b)), and round long particles are covered on the surface of the MPS/interlayer/TCP-AlSi-A granules (Figure 3 (c)).

From the EDX analysis shown in Figure 4, the peaks of Ca, P, Si and Al are found on the TCP-AlSi-A granules (Figure 4 (a)), however the peaks of Ca, P and Al may disappear on the interlayer/TCP-AlSi-A granules (Figure 4 (b)), suggesting that Si-O interlayer entirely covered on the TCP-AlSi-A granules by rf-magnetron sputtering. The Si, Ca, P and Al peaks are found on the MPS/interlayer/TCP-AlSi-A granules, and the peak intensity of Si is stronger than that of Ca, which may be due to the formation MPS coating layer on the granules.

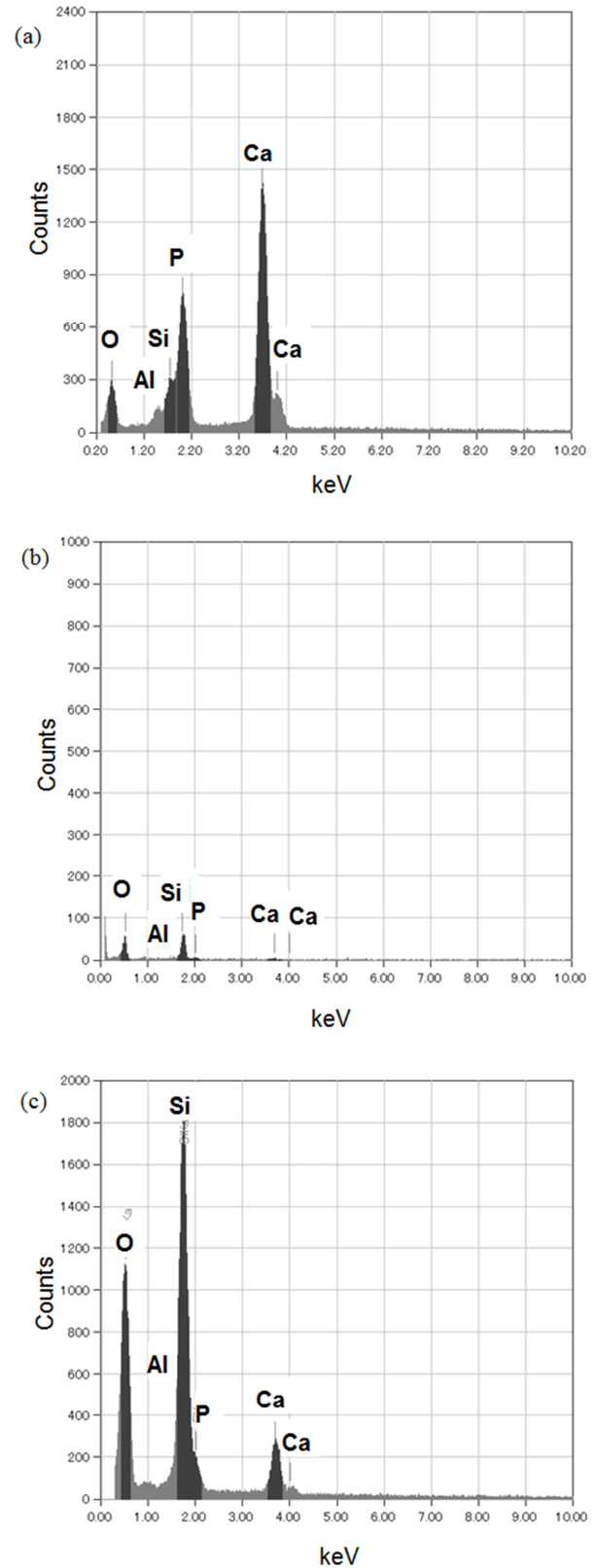


**Figure 3.** SEM images of TCP-AlSi-A (a) and interlayer/TCP-AlSi-A (b) and MPS/interlayer/TCP-AlSi-A granules (c). TCP-AlSi-A granules were obtained by heating at 1100 °C.

Figure 5 shows the pore size distribution (log differential pore volume distribution) of TCP-AlSi-A granules (a) and MPS/interlayer/TCP-AlSi-A granules (b). The TCP-AlSi-A granules do seem to have few pores in the range of 1 nm to 100 nm, while a peak of pore distribution are seen at around 2-3 nm in size on/in the MPS/interlayer/TCP-AlSi-A granules, suggesting the coating layer of MPS particles embedded on the interlayer/TCP-AlSi-A granules by dipping the solution containing TEOS, nitric acid and CTAB, drying and heating at 500°C.

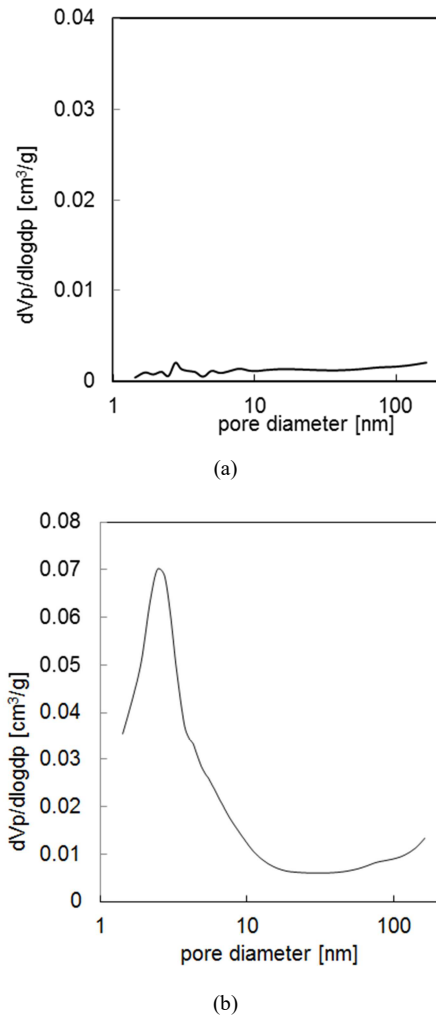
Figure 6 shows the bright field image of the high resolution transmission electron microscope and the SAED profiles of the MPS/interlayer/TCP-AlSi-A granules. The area A is the particles embedded on the granules, and the area B is the granules. The d-spacing of 8.1 Å at the area B may be assigned with the plane of β-TCP (1012) and HAp (1010), indicating that the granules are composed of β-TCP and partially formed HAp. The d-spacing value of 2.8 Å at the area A and B may correspond to the plane of SiO<sub>2</sub> (0002),

HAp (3030) and HAp (1122), suggesting a formation of silicate layer on the granules.

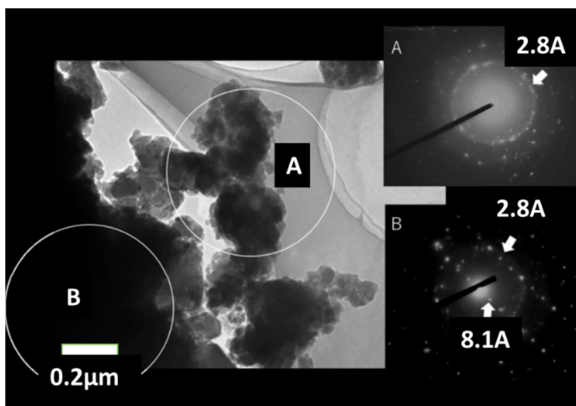


**Figure 4.** EDX profiles of TCP-AlSi-A (a), interlayer/TCP-AlSi-A (b) and MPS/interlayer/TCP-AlSi-A granules (c). TCP-AlSi-A granules were obtained by heating at 1100 °C.





**Figure 5.** Pore size distribution (Log differential pore volume distribution) of TCP-AlSi-A granules (a) and MPS/interlayer/TCP-AlSi-A granules (b).

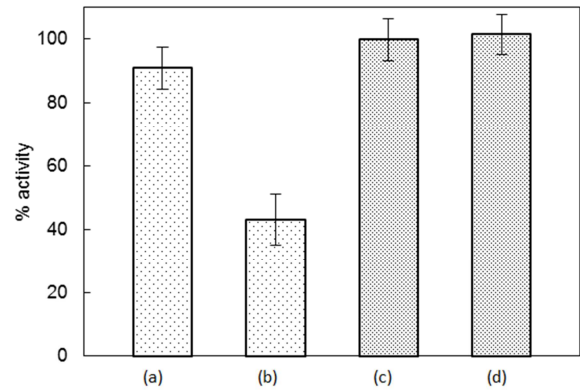


**Figure 6.** Shows the bright field images and the SAED profiles of the surface area of MPS/interlayer/TCP-AlSi-A granule.

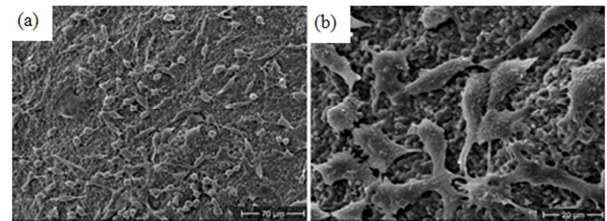
### 3.2. Cytocompatibility

Figure 7 shows the results of the Alamar blue (AB) assay, in which L929 cells were exposed to various materials ((a, b) TCP-AlSi-A granules and (c, d) MPS/interlayer/TCP-AlSi-A granules) by adding 0.1 mg (a, c) or 0.3 mg (b, d) into each well of a 96-well plate for 24 hours. When the concentration

of the TCP-AlSi granules was increased to 0.3 mg, the activity was remarkably reduced, while the activity was retained at a high level for the MPS-coated TCP-AlSi (MPS/interlayer/TCP-AlSi-A) granules. The morphologies of the L929 cells were stretched on the MPS/interlayer/TCP-AlSi-A granules, as shown in Figure 8.



**Figure 7.** Results of the Alamar blue assay, in which L929 cells were exposed to various materials ((a, b) TCP-AlSi-A granules and (c, d) MPS/interlayer/TCP-AlSi-A granules) by adding 0.1 mg (a, c) or 0.3 mg (b, d) into each well of a 96-well plate for 24 hours.



**Figure 8.** Morphologies of the L929 cells on the MPS/interlayer/TCP-AlSi-A granules obtained by adding 0.3 mg into a well of 96-well plates for 24 hours, observed at (i) low magnification and (ii) high magnification.

The TCP-AlSi-A granules and MPS/interlayer/TCP-AlSi-A granules demonstrated good biocompatibility, according to the results of the AB assay. Previously, we reported upon MPS-coated TCP granules that demonstrated high protein-adsorption capability [12]. The amount of adsorbed protein on the MPS/interlayer/TCP granules was 2.3 times higher than that on the TCP granules. The proteins adsorbed on the materials may provide favorable surfaces for cell adhesion and proliferation.

## 4. Conclusion

TCP granules containing  $\text{Al}_2\text{O}_3$  and  $\text{SiO}_2$  were formed using alginate, and heated from 1100 to 1300°C. During heating at 1300°C, no phase transformation from the  $\beta$ -TCP phase to the  $\beta$ -TCP phase was observed, and the compressive strength was remarkably improved. A Si-O interlayer was formed by reactive rf-magnetron sputtering on the  $\beta$ -TCP granules containing  $\text{Al}_2\text{O}_3$  and  $\text{SiO}_2$ , and the MPS layer was successfully coated onto the obtained interlayer/TCP-AlSi-A granules. The MPS-coated interlayer/TCP-AlSi-A granules demonstrated good biocompatibility, as determined from the

results of Alamar blue assay and SEM observations of the morphologies of the cells.

## Acknowledgements

The authors are grateful to Dr. T. Inamura for carrying out BJH pore size distribution measurements at the Technology Research Institute of Osaka Prefecture

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