Improving the biocompatibility of tobermorite by incorporating calcium phosphate clusters

Revised manuscript

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BACKGROUND: In our earlier work, tobermorite containing calcium phosphate (CP) clusters (CP-Tob) was hydrothermally prepared in the $CaO–SiO_2–P_2O_5–H_2O$ system for biomedical applications.

OBJECTIVE: CP-Tob was used to investigate the influence of CP cluster incorporation

on its biocompatibility.

METHODS: Tobermorite samples with and without CP clusters were hydrothermally prepared at 180 °C for 40 h. The biocompatibility, structure, and density of states of the tobermorite samples were investigated by experimental and first principles methods.

RESULTS: The amounts of lysozyme and bovine serum albumin adsorbed on CP-Tob were higher than those on tobermorite without CP clusters. Cluster incorporation caused a decrease in the solubility, resulting in the enhancement of the cell compatibility. The calculated results indicated that incorporating clusters, which interact with the silicate units of tobermorite, led to a change of the density of states of tobermorite.

CONCLUSIONS: Incorporation of CP clusters in tobermorite led to improvement of the biocompatibility evaluated by biological and computational analyses.

1. Introduction

Several types of inorganic osteogenic ions released from bioactive glass have been suggested to improve bone formation on the surface of biomaterials by gene activation [1,2]. Calcium ion is a crucial element of bone. Many researchers have reported that silicate ions released from materials enhance their bone forming ability [3,4]. Calcium silicates show great potential as bone substitutes because they release osteogenic ions, and many studies regarding biomedical applications have been reported [5–7].

Tobermorite is a crystalline calcium silicate hydrate formed by a stacking assembly of CaO polyhedral layers linked on both sides to silicate chains in the c direction, resulting in the formation of nanospaces in the interlayer region containing exchangeable cations and water molecules [8]. Tobermorite shows hydroxycarbonated apatite forming ability in simulated body fluid [9]. It has also been reported that Ag^{+} and Zn^{2+} ions can be incorporated into the structure of tobermorite by ion exchange to give tobermorite antimicrobial properties [10].

We have successfully prepared a new type of tobermorite for biomedical applications by incorporating calcium phosphate (CP) clusters into its structure using a hydrothermal process [11]. Cluster incorporation is assumed to cause a change of the electronic states of tobermorite. There is little information available about the relationship

between the biocompatibility and the density of states of materials. In the present work, the effect of incorporating clusters into tobermorite on the biocompatibility was investigated using experimental and computational methods.

2. Materials and methods

A slurry consisting of quartz, calcium hydroxide, sodium dihydrogen phosphate, and dilute hydrochloric acid with a 10/3 solvent/solid ratio was hydrothermally treated at 180 °C for 40 h to prepare CP-cluster-containing tobermorite (CP-Tob). Pure tobermorite was prepared as a control under the same hydrothermal treatment using a slurry consisting of quartz, calcium hydroxide, and distilled water as a solvent (hereafter denoted by Tob). The specific surface areas of CP-Tob and Tob determined by nitrogen gas sorption analysis were almost the same (around 40 m^2/g).

Lysozyme (LSZ, assay min. 80%) and bovine serum albumin (BSA, assay min. 99%) were used as model proteins to evaluate the adsorption abilities of the samples. A 0.5 mg/mL solution of each protein was prepared with phosphate buffer solution (54 mM disodium hydrogen phosphate and 13 mM potassium dihydrogen phosphate) at pH 7.4. The samples were soaked in the solution with 10/3 solid/liquid mass ratio for 30 min. After soaking, the supernatants were removed by centrifugal separation and the LSZ and

BSA amounts in them were evaluated using an ultraviolet spectrometer by the adsorption band at 280 and 595 nm, respectively. To evaluate the solubility of the samples at the initial stage of soaking, the sample powders were soaked with a 10/3 solid/liquid mass ratio in Tris-buffer solution (50 mM $(CH_2OH)_3CNH_2$ and 45 mM HCA) at pH 7.4, which was used for simplicity. The concentrations of calcium and silicate ions released from the samples were determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES). The pH after soaking was monitored using a pH meter. Osteoblast-like cells (MC3T3-E1 cells) were used to investigate the cell compatibility of the samples as a primary cell test. The samples were uniaxially pressed at 25 MPa in a stainless steel die to prepare pellets with 13 mm diameter. The cell suspensions with 6×10^4 cells/well were seeded onto these samples after sterilization with ethylene oxide gas in a 12 well plate. All of the cultures were supplemented with alpha minimum essential medium containing 10% fetal bovine serum and incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 for 5 days. After culturing, the cells on the samples were fixed by 2.5% glutaraldehyde for 40 min at 4 °C, dehydrated using a series of increasing concentration ethanol solutions, and finally dried with hexamethyldisilazane. The surfaces of the dried samples were observed by scanning electron microscopy (SEM).

The present calculations were performed using first-principles projector

augmented-wave code Quantum MAterials Simulator (QMAS) [12] to obtain the stable configurations and density of states of CP-Tob clusters using a 3×2×1 supercell. The periodic cell with 11.69 Å of a-axis, 7.37 Å of b-axis and 22.88 Å of c-axis in size was used based on our XRD results. Only the Γ point was used as the first step of the calculation. The cluster, whose lowest energy structure has D_{3h} symmetry [13], was located in the interlayer space of tobermorite without exchangeable cations and water molecules based on our previous experimental results [11]. Considering the size of the interlayer space of tobermorite, the major axis of the cluster was selected to be parallel to the *c* axis.

3. Results and discussion

In our previous work, tobermorite showed specificity for LSZ adsorption in phosphate buffer solution [14]. Figure 1 shows the amounts of LSZ adsorbed on the samples after soaking in the LSZ solution. Both of the two samples show similar LSZ adsorption behavior; immediately after the beginning of soaking (0–10 min), the amounts of LSZ adsorbed on the samples markedly increased, and then the adsorbed amounts stabilized. Note that CP-Tob shows two-times higher LSZ adsorption ability than Tob. On the other hand, the BSA adsorption ability tests resulted that CP-Tob had a higher BSA

adsorption ability (27 mg/g) than that (0 mg/g) of Tob after 30 min of the soaking. These indicates that incorporating CP clusters in tobermorite leads to enhancement of the adsorption ability for LSZ and BSA.

The amounts of calcium and silicate ions released from CP-Tob into the Tri-buffer solution after soaking for 4 d were less than those released from Tob, as shown in Figure 2. The pH values of the Tris-buffer solutions after soaking Tob and CP-Tob for 4 d were determined to be 7.54 and 7.41. Calcium silicates show a tendency to increase the pH because of their dissolution, leading to an inflammatory response after implantation. Incorporation of CP clusters into tobermorite lead to a decrease in the solubility, resulting in suppression of the pH increase.

Figure 3 shows SEM photographs of the pellet surfaces after 7 days of incubation. Numerous cells attached and spread on the CP-Tob surface. In contrast, several round-shaped cells are observed on the Tob surface. The silicon concentration, which induces cell activation, has not been optimized. Osteosarcoma-derived MG63 cells have been reported to dramatically decrease after culturing in a medium containing around 170 ppm silicon [15]. It has also been reported that supplementing the growth medium with 30 μmol/L silicon increases the proliferation of human osteoblast-like cells [16]. These findings indicate that the two types of tobermorite have the potential of enhancing cell

proliferation because of their appropriate silicon ion releasing abilities, as shown in Fig. 2. The color of the medium after culturing Tob for 3 days changed because of the increase in the pH. In contrast, the medium after incubating CP-Tob for 3 d was almost the same color as the initial culture color. Suppression of the pH increase by incorporating CP clusters improves the cell compatibility.

The calculation result showed that the CP cluster can stably exist in the interlayer spaces of tobermorite. The simulated structure of a CP cluster interacting with Q_{Si}^3 units of the silicate layer is shown in Fig. 4, and for this configuration, the partial density of states of hydrogen elements of the Q_{Si}^3 units, and oxygen and calcium, phosphorous elements of the cluster in the interlayer space of tobermorite are shown in Fig. 5. Qsi^n, where n is the number of bridging oxygen atoms to a neighboring tetrahedron, shows bonding to SiO_4 tetrahedra. New electronic states formed near the Fermi-level by incorporating the CP cluster. The zeta potentials, evaluated by an electrophoretic light scattering method, of the samples after 1 min of soaking in the phosphate buffer solution were almost the same value between -25 and -30 mV. We speculate that the formed states act as adsorption sites for LSZ and BSA, resulting in the enhancement of the adsorption ability. In the spectra of H, O, and P, two peaks are observed at the same energy levels, which are indicated by dotted lines in Fig. 5. This means that there is an interaction

between these elements. This interaction increases the bonding strength of the tobermorite structure, leading to a decrease in the solubility. Fourier transform infrared spectroscopy analysis has shown that the incorporation of clusters in tobermorite causes a change in the Si–O bonds of the Q_{Si}^3 units of tobermorite [11], which is consistent with the interaction obtained from the calculation results. This calculated structure is a preliminary structure as the first step to evaluate the structure and density of states of tobermorite incorporating CP clusters. To better understand the biocompatibility of CP-Tob, calculations with various initial structures and in the presence of water molecules are in progress.

4. Conclusions

The biocompatibility of CP-Tob was investigated to clarify the effect of incorporating CP clusters on the protein adsorption ability, cell compatibility, and solubility. The incorporation of CP clusters resulted in the formation of new energy states near the Fermi-level, leading to the enhancement of the LSZ and BSA adsorption abilities. Based on a calculation of the structure, the solubility of tobermorite decreased because of the formation of an interaction between the clusters and the Q_{Si}^3 units of tobermorite. As a result, the cell compatibility of tobermorite was enhanced by incorporating the CP

clusters. These results suggest that incorporation of clusters into tobermorite is a potential approach to improve its biocompatibility and chemical stability.

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Figure captions

Figure 1. LSZ adsorption behavior on Tob (○) and CP-Tob (●) after soaking in phosphate buffer solution.

Figure 2. Ca^{2+} and Si^{4+} ion concentrations in Tris-buffer solution from after soaking Tob and CP-Tob for 4 days.

Figure 3. SEM images of (a) Tob and (b) CP-Tob surfaces after culturing MC3T3-E1 cells for 7 days.

Figure 4. Simulated structure of the CP cluster interacting with silicate units (Q_{Si}^3).

Figure 5. Partial densities of state (PDOS) of CP-Tob. The labels for atoms are the same as in Fig. 4. The Fermi level is chosen as zero energy.

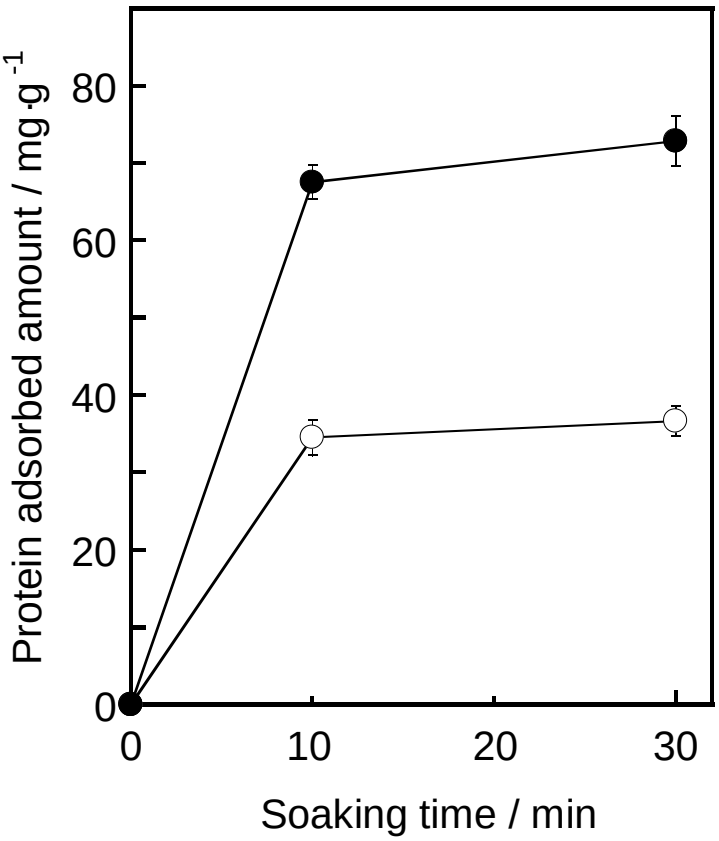
Protein adsorbed amount / mg·g-1
80
60
40
20
0
0
10
20
30
Soaking time / min

Fig. 1

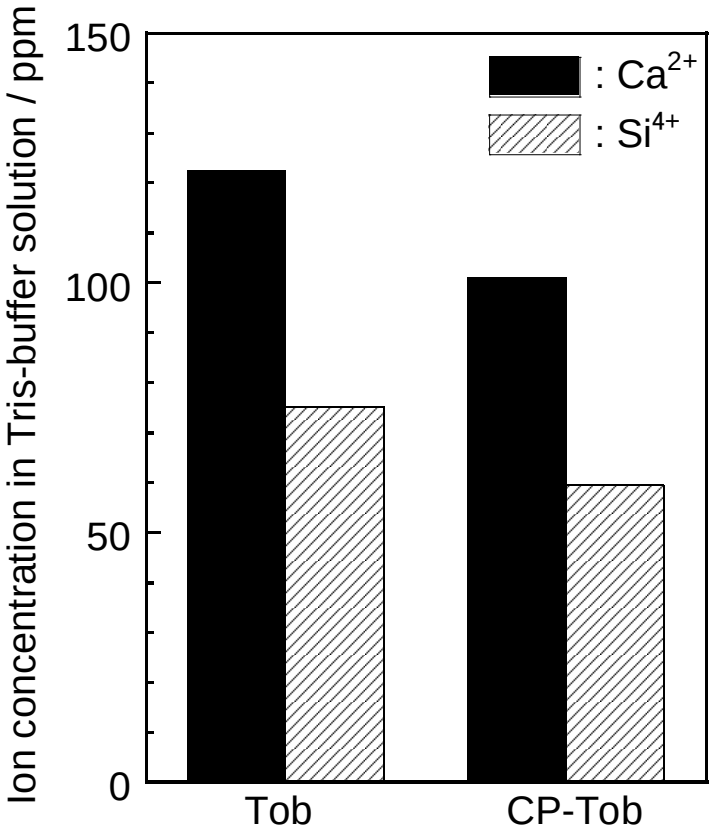
Ion concentration in Tris-buffer solution / ppm
150
100
50
0
: Ca^{2+}
: Si^{4+}
Tob
CP-Tob

Fig. 2

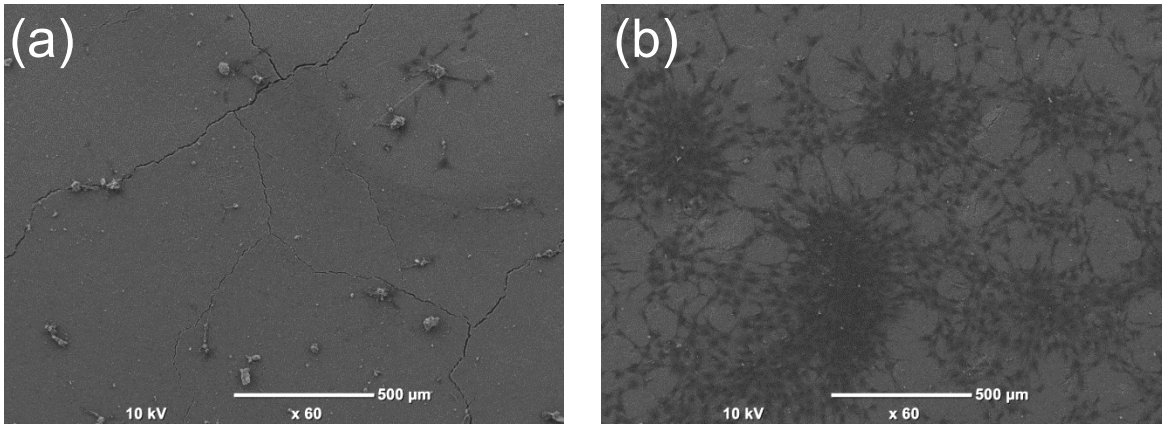

Fig. 3

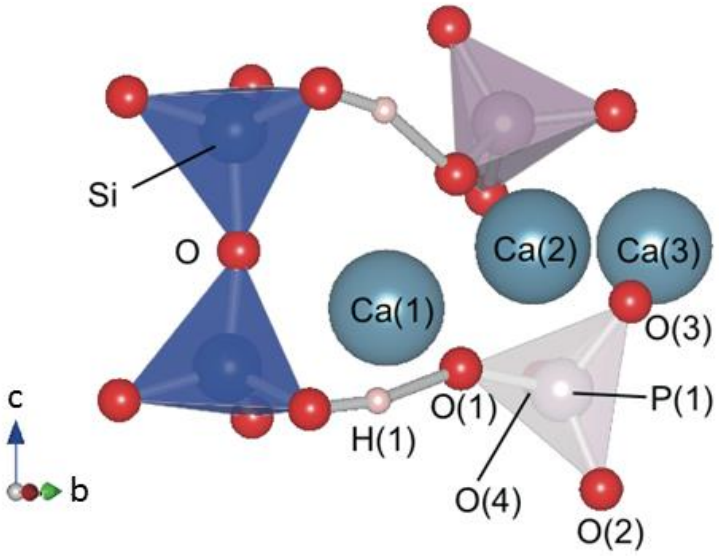

Fig. 4

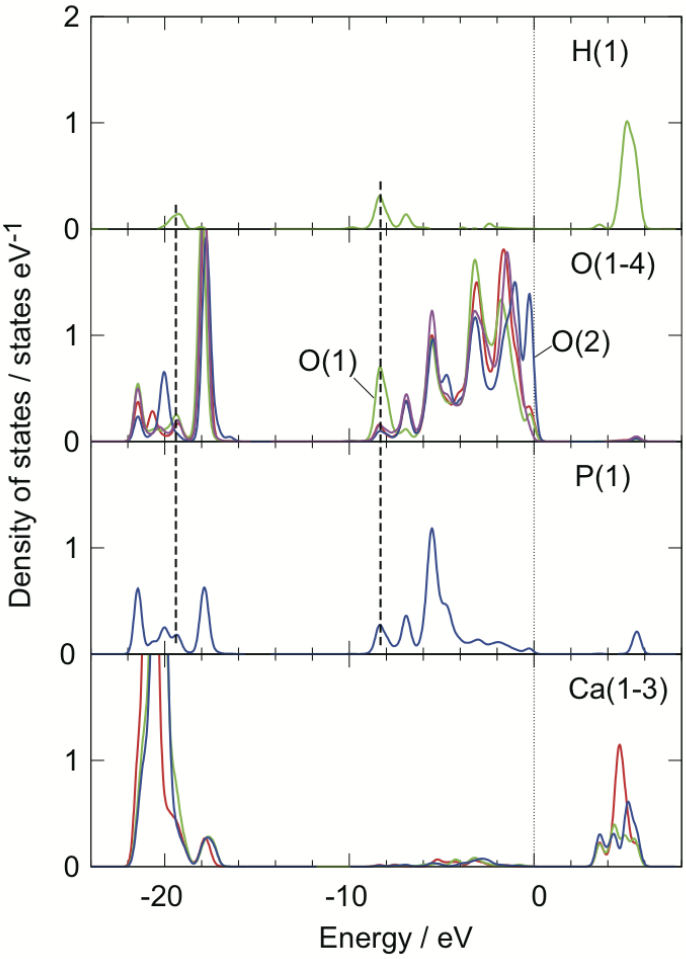
H(1)
O(1-4)
O(1)
O(2)
P(1)
Ca(1-3)
Density of states / states eV-1
Energy / eV
2
1
0
-20
-10
0

Fig. 5