Fabrication of Sr-substituted hydroxyapatite ceramics with different anisotropic structures and their osteodifferentiation

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Abstract

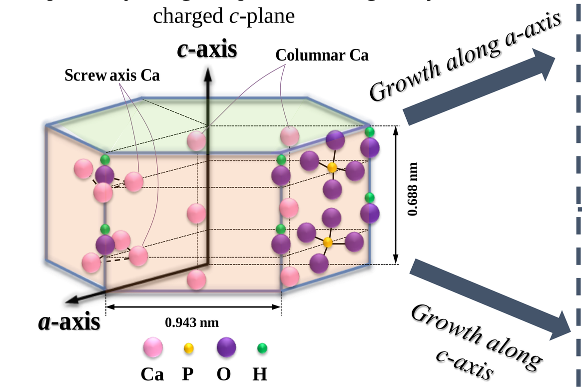
Hydroxyapatite (HAp) has two kinds of crystal planes, which are the *a*-plane and *c*-plane. If one can control the preferred orientation of apatite compounds, the resulting textured ceramics may exhibit novel functions. On the other hand, it is known that strontium activates osteoblasts and inhibits the resorption of osteoclasts. An aim of this study is to create the anisotropically-controlled Sr-substituted HAp ceramics (SrHAp), and to clarify the effect of the crystal plane on osteoblasts by culturing cells on the materials. In this study, we fabricated the textured SrHAp, and examined the osteogenic differentiation ability using rat bone marrow stromal cells.

1. Introduction: Background and aim

Hydroxyapatite ($(Ca_{10}(PO_4)_6(OH)_2$; HAp)

Main inorganic components of biological bone

A positively-charged *a*-plane and a negatively-charged *c*-plane



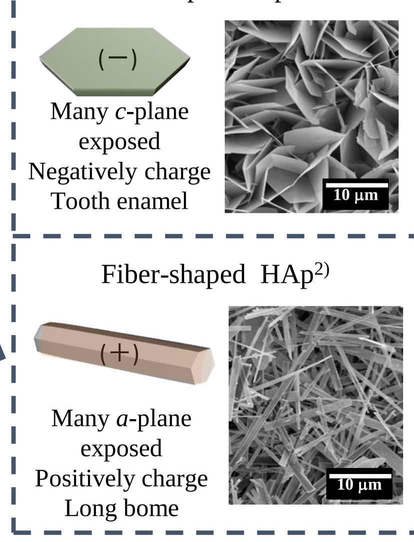
Anisotropic control

Plate-shaped HAp[1)]

Many *c*-plane exposed
Negatively charge
Tooth enamel

Fiber-shaped HAp[2)]

Many *a*-plane exposed
Positively charge
Long bone

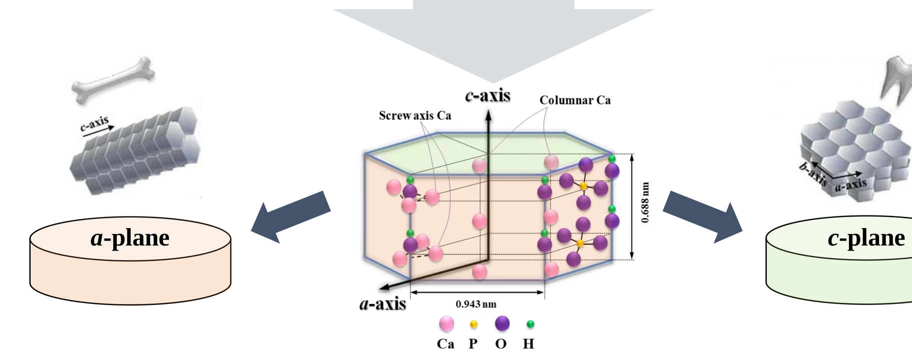


Control of anisotropy makes it possible to produce materials with specific properties

1) Z. Zhuang et al., Mater. Sci. Eng. C, 33, 2534-2540 (2013). 2) M. Aizawa et al., Biomaterials., 26, 3427-3433 (2005).

In vivo long bones expose more of *a*-plane
Tooth enamel is mostly exposed on *c*-plane
→ **Biological bone is anisotropic!**

Effect of each crystal plane of HAp on the cell is still unclear



To create a model material (apatite ceramics) with controlled anisotropy and to elucidate the relationship between the anisotropy of the material and the function of the cell.

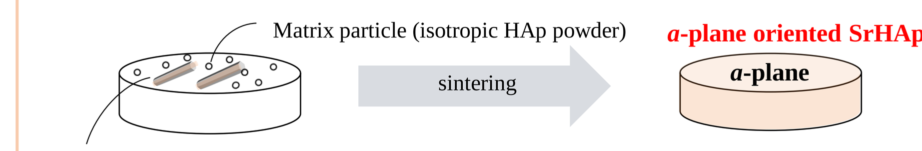
Preparation of strontium-substituted apatite ($(Ca, Sr)_{10}(PO_4)_6(OH)_2$; SrHAp) with well-controlled anisotropy

SrHAp with a large number of exposed *c*-plane

Successful preparation of *c*-plane oriented SrHAp by reactive template grain growth method using hexagonal α-tristrontium phosphate (α-$Sr_3(PO_4)_2$; α-TSP) particles[3)]

SrHAp with a large number of exposed *a*-plane

Successful preparation of *a*-plane oriented SrHAp by template grain growth method[4)] using fibrousstrontiumapatite ($Sr_{10}(PO_4)_6(OH)_2$: SrAp)



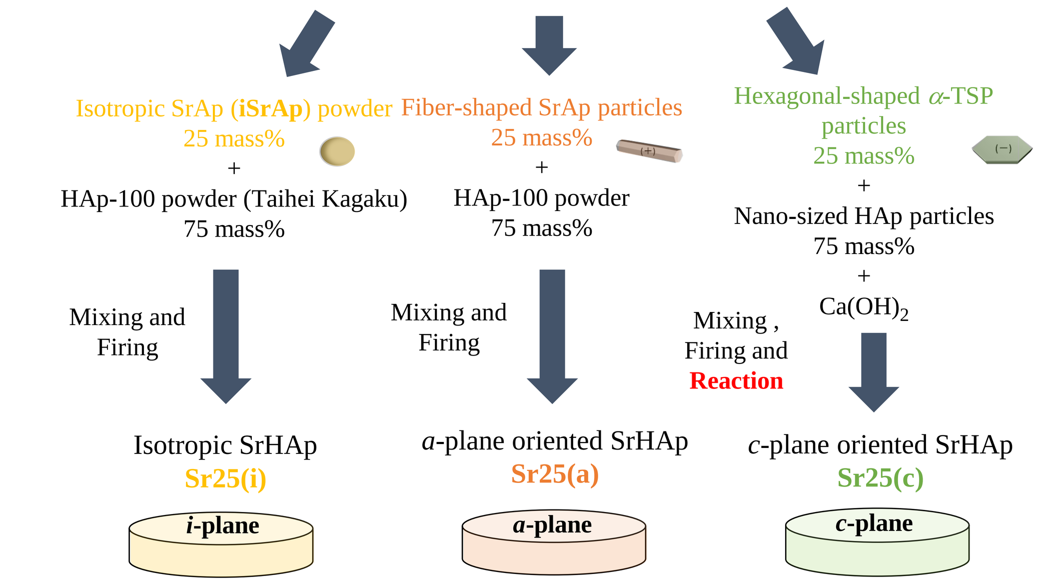
Template particles (SrAp fiber) ··· Anisotropic shape, preferably monodisperse

Template particles incorporate the matrix particles and grow in an orientated manner.

In addition to the above two ceramics, isotropic SrHAp will be produced and evaluated in vitro to elucidate the relationship between anisotropy and osteoblasts.

3) S. Yoshida et al., J. Asia Ceram. Soc., 8, 57-64(2020).
4) T. Tani, J. Ceram. Soc. Jpn., 114, 363-370 (2006).

Three types of the SrHAp ceramics with various anisotropic structures



2. Preparation of three types of starting powders

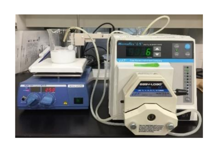
Fiber-shaped SrAp particles

Starting solution (400 cm³)
pH = 2.50
$SrCl_2$ 0.167 mol·dm⁻³
$(NH_4)_2HPO_4$ 0.100 mol·dm⁻³
$(NH_2)_2CO$ 0.500 mol·dm⁻³
conc. HCl pH adjustment

↓ Heating and stirring in an oil bath 80 °C, 90 rpm, 24 h
↓ Heating in an oil bath 90 °C, 144 h
↓ Suction filtration and drying
↓ **Fiber-shaped SrAp particles**

iSrAp powder

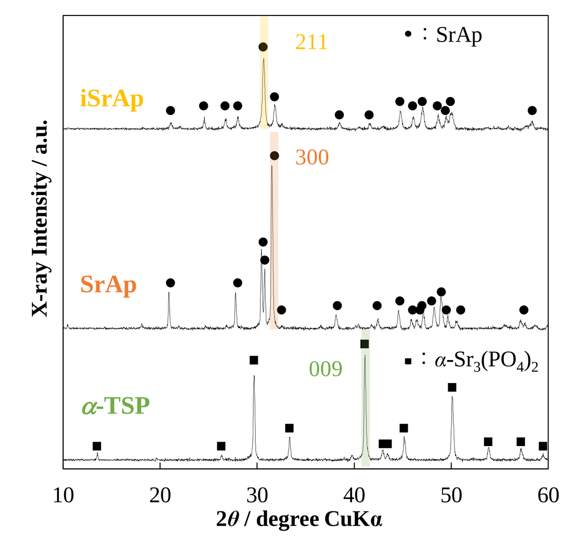
Starting solution
$Sr(NO_3)_2$ 0.0676 mol·dm⁻³
↓ Dripping 6 cm³ / min
H_3PO_4 0.0405 mol·dm⁻³
↓ Adjusted to pH 10 (Ammonia water)
↓ Stirring and aging 600 rpm, 72 h
↓ Suction filtration
↓ Calcination
Under steam atmosphere
Holding temperature: 800 °C
Holding time : 1 h
Heating rate : 5 °C · min⁻¹
↓ Grinding with mortar and pestle
↓ **iSrAp powder**

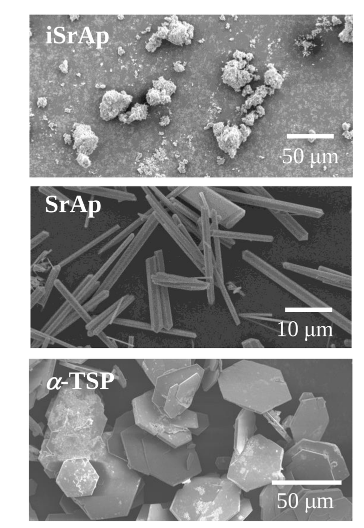


α-TSP particles[5)]

Starting solution (500 cm³)
$Sr(CH_3COO)_2$ 0.0167 mol/dm³
$(NH_4)_2HPO_4$ 0.0100 mol/dm³
$(NH_2)_2CO$ 0.0500 mol/dm³
HNO_3 0.0480 mol/dm³
↓ Heating in an oil bath 80 °C, 24 h
↓ Suction filtration and drying
Solution and powder are separated once
Solution is used for hydrothermal synthesis
↓ α-strontium hydrogen phosphate (α-$SrHPO_4$) particles
↓ Planetary ball mill: 2 mmϕ ZrO_2 180 g
Wet grinding
Solvent: pure water 40 cm³
Grinding time: 1 h
Rotation speed: 300 rpm
↓ Suction filtration and drying
↓ Grinded α-$SrHPO_4$ particles
↓ Hydrothermal treatment 150 °C, 3 h Rotation speed: 150 rpm
↓ Suction filtration and drying
↓ **α-TSP particles[5)]**

5) A. Ishida et al., J. Eur. Ceram. Soc., 40, 351-357(2017).





・The (300) plane, which corresponds to the *a*-plane, is the strongest line of the SrAp particle and has a fibrous morphology.

・The (009) plane, which corresponds to the *c*-plane, is the strongest line in the α-TSP particles, and the particles were found to have a hexagonal plate shape with little agglomeration.

3. Fabrication of SrHAp ceramics with anisotropic structures and their properties

Fabrication of Sr25(a) and Sr25(i) ceramics

Mixing powder (each 0.5 g)
Fibrous SrAp or iSrAp powder
HAp-100 powder (Taihei-kagaku, Japan)

Mixing ratios

Sample	SrAp mass%	iSrAp mass%	HAp mass%
Sr25(a)	25		75
Sr25(i)		25	75

↓ Mixing in a micro V-type mixer (Sr25(a)) 30 min
Mortar grinding (Sr25(i))
↓ Uni-axial pressing
Pressure：100 MPa
Diameter：15, 14 mmϕ
↓ Green compact
↓ Firing
In steam atmosphere
Firing temperature: 1300 °C
Holding time : 5 h
Heating rate: 5 °C·min⁻¹
↓ Ceramics (i-plane, a-plane)

Fabrication of Sr25(c) ceramics

Mixing powder (0.5 g)
・Template particles : α-TSP particles
・Matrix particles : Nano-sized HAp particles
・Reaction complementary agents : $Ca(OH)_2$

$3Sr_3(PO_4)_2 + Ca(OH)_2 \rightarrow CaSr_9(PO_4)_6(OH)_2$

➢ Weighing to stoichiometry

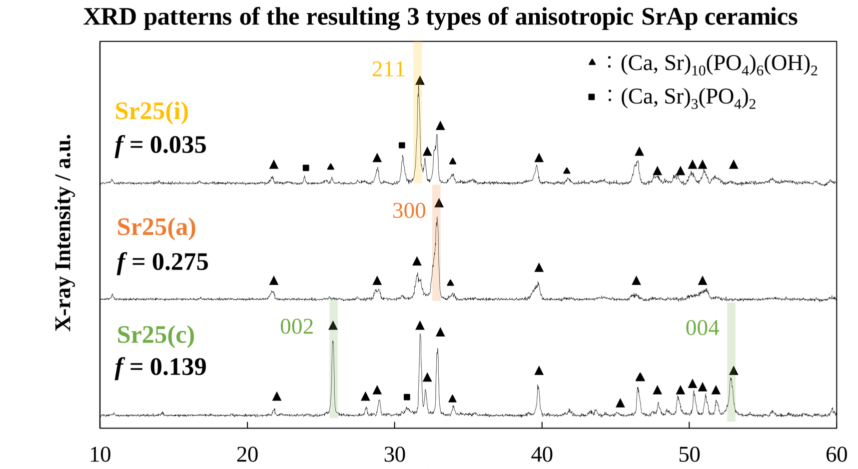
Nano-sized HAp / α-TSP particles
Deposition of nano HAp particles on α-TSP particles at 75 mass% of final product
α-TSP particles: 25 mass%
Nano-sized HAp particles : 75 mass%

↓ Mixing in a micro V-type mixer 30 min
↓ Uni-axial pressing
Pressure：100 MPa
Diameter：14 mmϕ
↓ Green compact
↓ Firing
Under steam atmosphere
950 °C, 10 h
1300 °C, 2 h

Firing conditions: 950 °C, 10 h (Solid-state reaction) → 1300 °C, 2 h (Grain growth Sintering) → Cooling

↓ Ceramics (c-plane)

XRD patterns of the resulting 3 types of anisotropic SrAp ceramics



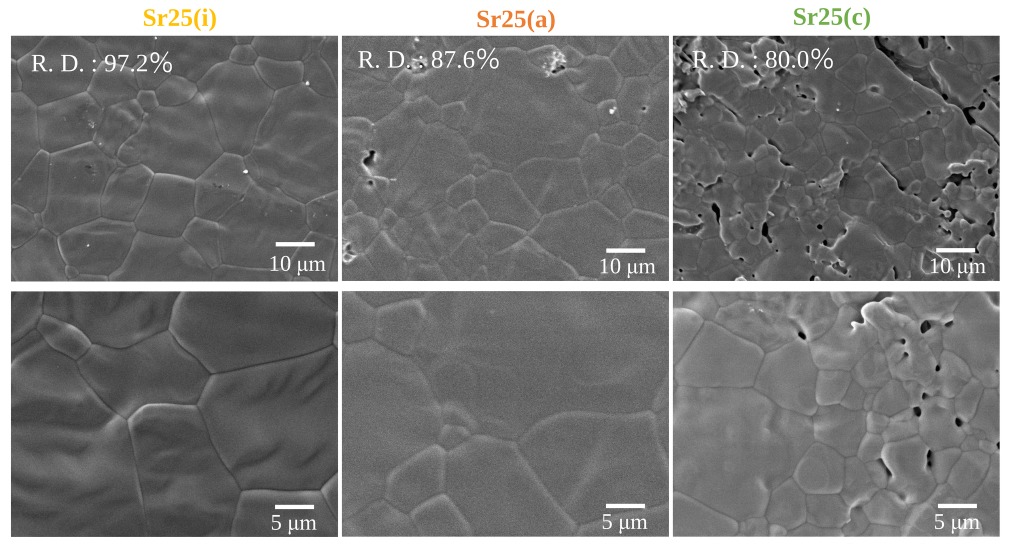
・All ceramics had apatite solid solution as the main crystalline phase.

・The higher the value of the orientation f calculated by the Lotgering method6), the more oriented the material is.

・The orientations of Sr25(a) and Sr25(c) ceramics are higher than those of Sr25(i), indicating that they expose more of the a- and c-planes, respectively.

6) F. K. Lotgering, J. Inorg. Nucl. Chem., 9, 113 (1959).

Microstructures of anisotropic SrAp ceramics

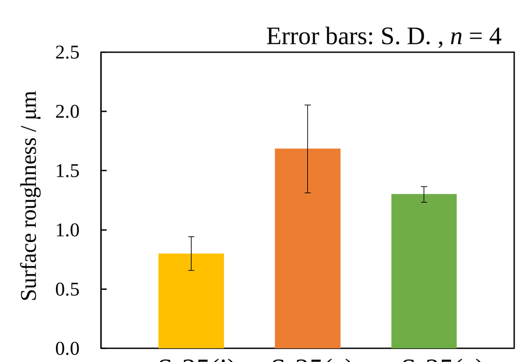


R.D. : Relative density

The Sr25(i) and Sr25(a) surfaces have a dense structure.
On the surface of Sr25(c), many pores were observed.
→ The pores on the surface of Sr25(c) were observed to be numerous, probably due to the formation of voids in the compact during the forming of the bulky platelets.

Chemical composition and surface roughness of anisotropic SrAp ceramics

Sample	Ca / mass% expected	Ca / mass% measured	Sr / mass% expected	Sr / mass% measured	Ca : Sr molar ratio	(Ca+Sr)/P
Sr25(i)	29.92	31.79	14.80	15.23	8.20 : 1.80	1.71
Sr25(a)	29.92	29.90	14.80	16.33	8.00 : 2.00	1.68
Sr25(c)	31.81	32.98	14.00	14.85	8.29 : 1.71	1.64

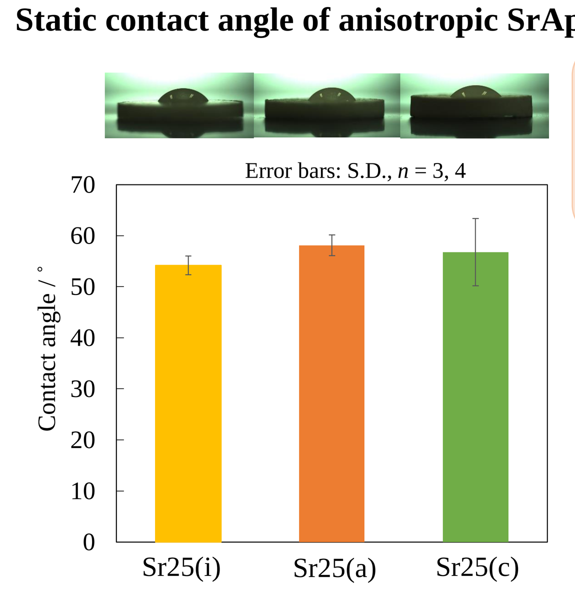


・The Ca and Sr contents were found to be close to the theoretical values.

・The Ca:Sr molar ratio shows that all ceramics contain the same amount of Sr.

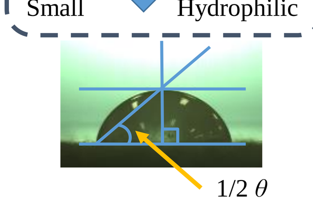
・The surface roughness was about 1 to 2 μm and there was no significant difference between the ceramics.
→ **Use for cell culture experiments**

Static contact angle of anisotropic SrAp ceramics



Conditions
Liquids: Ultra pure water
Discharge volume : 3.6 mm³
Humidity: 10 %
Room temp. : 20.9 °C
Calculation method : ATAN1/2θ

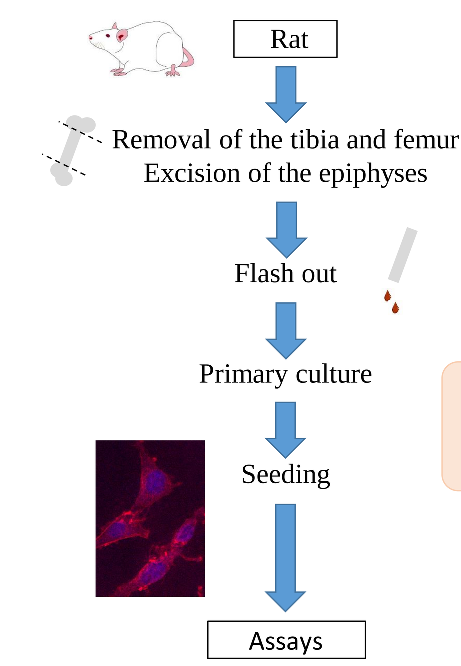
Contact angle	Wettability
Big	Hydrophobic
Small	Hydrophilic



・The contact angles of all the ceramics were around 50-60°, indicating that they had hydrophilic surfaces.
・In this study, the influence of the orientation of the ceramics on the wettability of the surface is considered to be small.

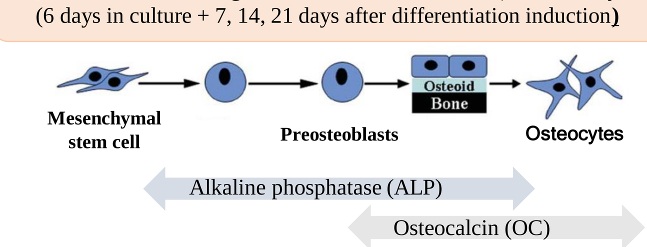
4. *In vitro* evaluation of SrHAp ceramics with anisotropic structures

***In vitro* evaluation using rat bone marrow-derived mesenchymal stem cells (RBMCs)**



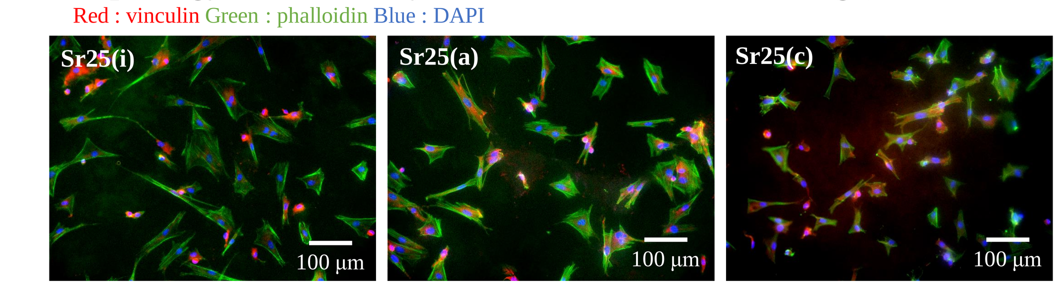
Culture conditions
Used cell: RBMCs
Used medium: α-MEM medium (+10% Fetal Bovine Serum)
+ 100 U·cm⁻³ Penicillin
+ 100 mg·cm⁻³ Streptomycin
Differentiation-inducing medium:
Normal medium
+ 10 mmol·dm⁻³ β- sodium glycerophosphate
+ 200 μmol·dm⁻³ L- ascorbic acid
+ 10 mmol·dm⁻³ Dexamethasone
Cell density : 1.0 × 10⁵ cells·cm⁻³
Culture substance : 48 well plate, Sr25(i), Sr25(a), Sr25(c)

Cell evaluations:
Immunofluorescence staining (24 h in culture)
Quantification of osteogenic differentiation markers (ALP activity, OC production)
(6 days in culture + 7, 14, 21 days after differentiation induction)

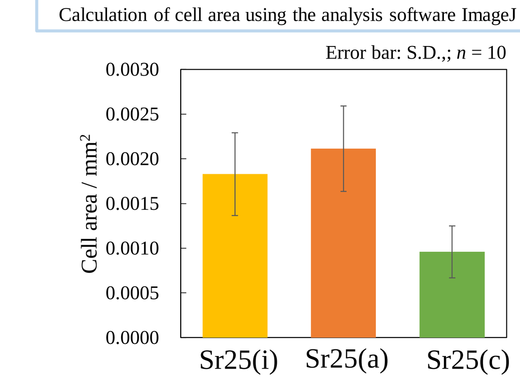


Morphology of the cells by immunofluorescence staining (24 h in culture)

Red : vinculin Green : phalloidin Blue : DAPI



Calculation of cell area using the analysis software ImageJ



・The staining results show that the cells on Sr25(a) and Sr25(i) are well spread.

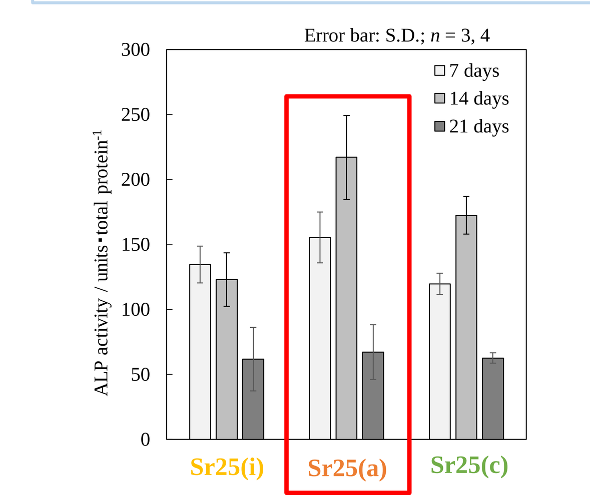
・In Sr25(c), more poorly elongated cells were observed than in the other ceramics.

・The calculation of the cell area showed that the cells on Sr25(a) had the highest cell area.

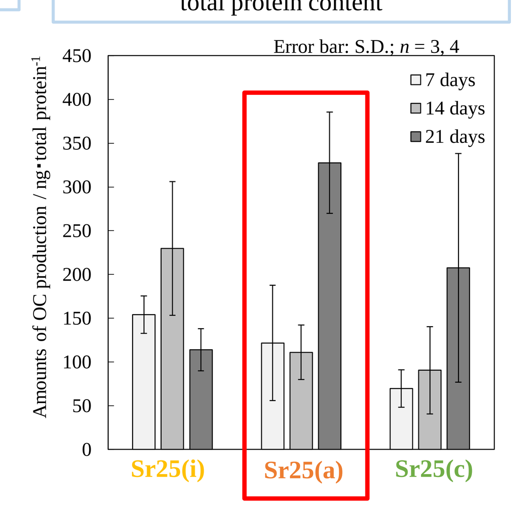
→ The Sr25(a) is considered to be a ceramic that promotes cell adhesion in the early stages of culture !

Determination of ALP and OC assays

ALP activity values normalised for total protein content



Amounts of OC production normalised for total protein content

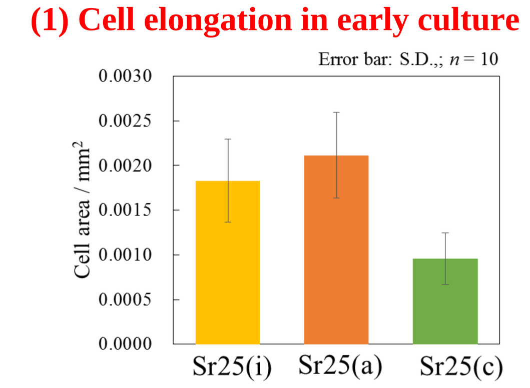


・ALP activity on Sr25(a) and Sr25(c) increased from day 7 to day 14.
The highest OC production on Sr25(a) and Sr25(c) was observed on day 21.

→**Sr25(a) showed the highest ALP activity on day 7 and 14 and the highest OC production on day 21 compared to other ceramics, suggesting that Sr25(a) is a good substrate for cell differentiation.**

Why did the *a*-plane of apatite promote osteoblast differentiation?

(1) Cell elongation in early culture

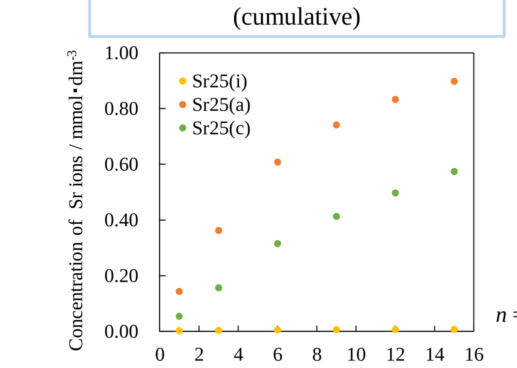


・The cell area on Sr25(a) is the largest.
・Bovine serum albumin (BSA) in FBS is selectively adsorbed to the *a*-plane orientated HAp and is thought to promote osteoblast adhesion and proliferation[7,8)].

→ **The *a*-plane of apatite may be better able to adsorb proteins and other substances that promote the adhesion and differentiation of osteoblasts.**

(2) Sr^{2+} ions eluted during incubation

Sr^{2+} ion elution in culture by ICP-AES (cumulative)



・Sr25(a) was found to have the highest concentration of eluted Sr^{2+} ions.

→**Sr^{2+} ions are known to be stimulants of osteogenesis[9)], and it is likely that the Sr^{2+} ions eluted in this study promoted osteodifferentiation.**

7) T. Hagio et al., Mater. Trans., 50, 734-739 (2009). 8) M.T. Bernards et al., Colloids Surf. B, 64, 236-247 (2008). 9) B. Kruppke et al., Dev. Growth Differ., 61, 166-175 (2019).

5. Conclusion

● **Fabrication of SrHAp ceramics with different anisotropic properties and their characterisation**

1) The main crystalline phase of the prepared ceramics was apatite solid solution.
2) The orientations *f* of Sr25(a) and Sr25(c) are higher than those of Sr25(i), indicating that they expose more of the *a*- and *c*-planes, respectively.
3) From the elemental analysis by ICP-AES, it was found that all the ceramics contained the same amount of Sr.

● ***In vitro* evaluation of SrHAp with different anisotropic properties using osteoblasts**

1) The calculation of the cell area showed that the cells on Sr25(a) had the highest cell area.
2) Compared to other ceramics, Sr25(a) showed the highest ALP activity at 7 and 14 days and the highest OC production at 21 days, suggesting that it is a favourable culture substrate for cell differentiation.

SrHAp ceramics with different anisotropy have been successfully fabricated. As far as we have investigated in this study, the a-plane of apatite is favourable for the promotion of osteoblast differentiation, and crystal anisotropy is one of the most important factors for the functional design of bioceramics.