Evaluation of the mechanical properties and biocompatibility of gypsum-containing calcium silicate cements

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Mineral trioxide aggregate (MTA) cement is widely used in the field of endodontic treatment. We herein synthesized calcium silicates from calcium carbonate and silicon dioxide, with the aim of reducing the cost associated with the MTA. Additionally, we prepared gypsum-containing calcium silicate cement to reduce the setting time while enhancing the mechanical strength. We evaluated the physical properties of this cement and investigated the response of human dental pulp stem cells (hDPSCs) grown in culture media containing cement eluate. Our results revealed that calcium silicates could be easily synthesized in lab-scale. Furthermore, we demonstrate that gypsum addition helps shorten the setting time while increasing the compressive strength of dental cements. The synthesized gypsum-containing calcium silicate cement showed minimal cytotoxicity and did not inhibit the proliferation of hDPSCs. These results suggested that the newly developed calcium silicate material could be a promising pulp capping material.

Keywords: Calcium silicate, Dental gypsum, Firing, Mineral trioxide aggregate cement, Pulp capping material

INTRODUCTION

The objective of endodontic treatment is to help maintain teeth in a healthy state and preserve their functionality1-6. In the field of endodontics, calcium-based cements are key materials that are used in a variety of procedures4-9. Mineral trioxide aggregate (MTA), a hydraulic cement that has gained popularity owing to its biocompatibility as well as antibacterial activities, is widely used in endodontic treatment4-6. However, MTA is a high-cost material and has issues such as a long setting time after the MTA has set, the setting reaction gradually proceeds to completion over the next several weeks4,26. MTA also contains gypsum, calcium sulfate dehydrate (setting promoter), and bismuth oxide (contrast agent)4,23. MTA is cured by the hydration of inorganic oxides; the hydration of calcium silicates within the MTA produces crystals of both calcium silicate hydrate, 3CaO·2SiO2·3H2O, and calcium hydroxide, Ca(OH)224,25. Reportedly, the mixing time of MTA should be less than 4 min26, with a setting time between 2.75 and 4 h26,27. After the MTA has set, the setting reaction gradually proceeds to completion over the next several weeks6,27.

We have focused this study on calcium silicates, that is the major component of MTA. We first synthesized calcium silicates from calcium carbonate and silicon dioxide and produced new MTA-like cement without Portland cement. Synthesized materials will be expected to enhance their clinical activity due to their availability with low-cost. Additionally, we prepared three kinds of gypsum-containing calcium silicate cements to reduce the setting time as well as to increase the mechanical strength. We evaluated the mechanical properties of these cements, as well as examined the biocompatibility of these new cements.

MATERIALS AND METHODS

Synthesis of calcium silicates

To prepare calcium silicates comprising MTA, the precursors, calcium carbonate (Guaranteed Reagent, FUJIFILM Wako Pure Chemical, Osaka, Japan) and silicon dioxide (Diatomaceous earth, Nacalai Tesque,
Kyoto, Japan) were mixed in an alumina crucible at a weight ratio of 5:1 according to the stoichiometric ratios obtained from the following chemical equation:

$$2\text{CaCO}_3 + \text{SiO}_2 \rightarrow \text{Ca}_2\text{SiO}_4 + 2\text{CO}_2$$

A 48 g quantity of the mixed powder of CaCO$_3$ and SiO$_2$ was then added to 50 mL of distilled water (DW) and mixed further. This test sample was sintered at 1,300°C for 1 h. After that, the sintered sample was crushed into a fine powder with an agate mortar. Then, an electric sieve shaker (MVS-1, AS ONE, Osaka, Japan) was used to separate out and collect particles with a size of \(\leq 75\ \mu m\). The powder with particles of 75 \(\mu m\) or less was used to prepare all samples used in the following experiments.

**X-ray diffraction analysis of synthesized calcium silicates**

X-ray diffraction (XRD) patterns of the calcium silicate powder were recorded using a multipurpose X-ray diffractometer (XRD; Ultima V, Rigaku, Tokyo, Japan) with a horizontal sample mount. The XRD patterns were collected in the diffraction angle (2\(\theta\)) range of 10–60°. We performed XRD analyses on three classes of sample: dried and sintered calcium silicate powder, sintered calcium silicate powder mixed with DW, and MTA (ProRoot MTA®, Dentsply Sirona, Ballaigues, Switzerland) powder mixed with DW acting as a control.

**Preparation of gypsum-containing calcium silicate cement**

In preliminary experiments, calcium silicate powder mixed with DW did not display satisfactory setting properties. We therefore added a dental plaster (\(\alpha\)-gypsum hemihydrate, Shimomura Gypsum, Saitama, Japan), to the sintered calcium silicate cement at different proportions of 25, 33, or 40% wt/wt; the corresponding samples were labeled as CS-P1, CS-P2, or CS-P3, respectively. This addition was performed to reduce the setting time of silicate cement and improve its mechanical strength. The experimental cement samples developed and used in this study are summarized in Table 1.

### Table 1  List of experimental cements and preparation conditions

| Sample Label | Cement                                | Water-to-Powder (w/p) ratio |
|--------------|---------------------------------------|----------------------------|
| P            | Dental plaster, gypsum dihydrate      | 0.27                       |
| D            | Dycal®, calcium hydroxide             | As per manufacturers’ instructions |
| M            | PRO ROOT MTA®                          | As per manufacturers’ instructions |
| CS           | Calcium silicate cement               | —                          |
| CS-P1        | Gypsum-containing calcium silicate cements | gypsum content: 25% | 0.5 or 0.4 |
| CS-P2        |                                       | gypsum content: 33%       | 0.5 or 0.4 |
| CS-P3        |                                       | gypsum content: 40%       | 0.5 or 0.4 |

**Measurement of the setting time of gypsum-containing calcium silicate cements**

The setting time of each cement sample was determined according to the JIS T 6605 standard corresponding to ISO 6873 1998. Briefly, each cement sample was mixed with DW at room temperature, then packed inside an acrylic ring (inter-diameter (ID): 10 mm, outer-diameter (OD): 16 mm, height: 5 mm) and placed on a glass plate. At the onset of mixing, a Vicat needle (weight: 200 g, diameter: 2 mm) was repeatedly dropped on the surface of the cement sample gently. The time at which a dropped needle produced no indentation was recorded as the setting time. As a reference, similar tests were carried out for several commercial calcium hydroxide-based pulp capping materials prepared with the manufacturers’ recommended water-to-powder (w/p) ratio. The commercial materials used were a commercial pulp-capping product (Dycal®, Dentsply Sankin, Tokyo, Japan), MTA, and the dental plaster which were labeled as cement D, cement M, and cement P, respectively. Three samples per experimental cement group were investigated, and the mean values are reported.

**Measurement of the compressive strengths of gypsum-containing calcium silicate cements**

After mixing samples CS-P1, CS-P2, and CS-P3 with prescribed amounts of DW, each cement was packed inside a Teflon mold (diameter: 6 mm, height: 12 mm) and placed on a glass plate to obtain cylindrical test specimens. After 24 h at room temperature, the compressive strength of each specimen was measured using a universal testing machine (Autograph AG-20KNXDplus, Shimadzu, Kyoto, Japan). The compressive strengths of cements P, D, and M used as references were also measured. In all cases, three samples of each cement group were evaluated.

**Preparation of cement-immersed cell culture media**

Immediately after mixing, samples of CS-P1, CS-P2, CS-P3, P, D, and M were individually packed inside acrylic rings (described above) and allowed to stand at room temperature for 2 h. After setting until the surface had hardened, each sample was immersed in 10 mL of \(\alpha\)-modified Eagle’s minimum essential medium without phenol red (\(\alpha\)-MEM) (GIBCO™, Thermo Fisher Scientific, USA) as a control.
Evaluation of the cytotoxicity of cement-immersed media

Samples of hDPSCs (Allcells, Emeryville, CA, USA), which had been subcultured 11–14 times in 10% fetal bovine serum (FBS)-containing α-MEM, were seeded within a 96-well plate at a density of 4,000 cells/well. The culture medium was changed to various cement-immersed experimental media, each containing eluates of a sample of CS-P1, CS-P2, CS-P3, P, D, or M, 6 h after seeding. After a 48 h-incubation period, the extracellular concentration of lactate dehydrogenase (LDH) released during the damage of cell membrane was measured. As reference, the concentration of LDH produced by hDPSCs that were cultured in normal α-MEM was measured. A cytotoxicity detection kit (TAKARA Bio, Otsu, Japan) was used for the measurement; the absorbance of each sample at 492 nm was recorded with a plate reader (Infinite Pro-200, Tecan Japan, Tokyo, Japan).

Live/dead staining assay

In order to evaluate the survival of cells in the cement-immersed experimental media, Live/dead staining was performed using LIVE/DEAD® Viability/Cytotoxicity Kit (Thermo Fisher Scientific) according to the manufacturer's instruction. The hDPSCs were cultured in the same manner as described above, in a 3.5-cm glass-bottom dish (Greiner Japan, Tokyo, Japan). After 48 h incubation, Calcein AM, which specifically stains living cells, and Ethidium homodimer-1 (EthD-1), which stains dead cells, were added to the culture to a final concentration of 1 µM and 2 µM respectively. After 30 min, the cells were observed under a microscope (Ti U, Nikon, Kawasaki, Japan).

Evaluation of the growth of hDPSCs in cement-immersed media

The hDPSC was seeded within a 96-well plate at a density of 4,000 cells/well. After 6 h following seeding, the medium was changed to a cement-immersed medium, as described above. After 48 h, an Alamar Blue® reagent (Thermo Fisher Scientific) was added to each well at a 1/10 volume ratio. After another 2 h, the fluorescence intensity (Ex: 560 nm, Em: 590 nm) was measured.

Alkaline phosphatase (ALP) activity staining

The hDPSC was seeded within a 48-well plate at a density of 12,000 cells/well. After 6 h seeding, the medium was changed to a cement-immersed medium and cells were grown for 7 days. The cells were then fixed in place using 4% paraformaldehyde (PFA) (Nacalai Tesque) phosphate buffered saline (PBS). After washing twice with 25 mM tris-buffered saline (TBS) with a pH of 7.4 (Bio-Rad Laboratories, Hercules, CA, USA) and further washing with 25 mM TBS with a pH of 10.0, ALP activity staining was performed using the NBT/BCIP reagent kit (Nacalai Tesque).

Statistical analysis

Unless otherwise specified, all experiments were repeated at least twice; in all cases similar results were obtained. To investigate the statistical significances of the data, an analysis of variance (ANOVA) was carried out with the KareidaGraph software (ver. 4.0, Hulinks, Tokyo, Japan). Scheffé’s multiple comparison test was then used; a p-value <0.05 was considered statistically significant.

RESULTS

XRD analysis of calcium silicate powder

The XRD pattern of the calcium silicate sample synthesized in this work shows characteristic peaks of calcium silicates (Ca₂SiO₄ and Ca₃SiO₅) (Fig. 1A). However, the peaks of calcium oxide (CaO) were also observed for the sintered calcium silicate powder. The formation of calcium hydroxide was observed after the sintered calcium silicate powder was mixed with DW (Fig. 1B). This Ca(OH)₂ peak was also observed in the XRD pattern of cement M mixed with DW (Fig. 1C). In
addition, peaks of various calcium silicates (CaSiO₃, Ca₂SiO₄, and Ca₃SiO₅) were observed in the calcium silicate cement and cement M at 2θ=18° and ~33–34° (Figs. 1B and C); additionally, a peak of bismuth oxide, added as the contrast agent, was also observed in cement M at 2θ=28° (Fig. 1C).

Investigation of the setting time of gypsum-containing calcium silicate cements
All CS-P1, CS-P2, and CS-P3 samples hardened after a much shorter setting time than that required for the setting of cement M; samples were prepared at a w/p ratio of 0.5 (Fig. 2A) and 0.4 (Fig. 2B) for CS-P cements added with gypsum. The setting time of these CS-P cements was about 35–60 min. Setting was slightly faster at w/p=0.4. Furthermore, the setting time was shortened for the CS-P3 cement group (Figs. 2A and B), which had the highest percentage of gypsum hemihydrate in the CS-P groups (Table 1). Based on the above findings, each of the three types of CS-P cements was then mixed at w/p=0.4 in subsequent experiments; the corresponding samples are labeled as CS-P (0.4). A single face of the CS cement sample required 24 h or more to set (data not shown).

Investigation of the compressive strength of gypsum-containing calcium silicate cements
In comparison to the CS cement, the compressive strengths of all three types of CS-P (0.4) cements were significantly increased by the addition of gypsum hemihydrate. The compressive strength of cements CS-P (0.4) 1-3 increased proportionally with the amount of gypsum hemihydrate added to the cements (Fig. 3).

pH of cement-immersed media
Each of the experimental cements listed in Table 1 was immersed in α-MEM (pH 7.6) for 24 h described above. The resulting pH was measured by a pH meter; these results are summarized in Table 2.

Cytotoxicity of cement-immersed media to hDPSCs
After 48 h of incubation, the amount of LDH released into the media from hDPSCs increased significantly for the sample exposed to cement D. Conversely, the levels of LDH leakage in media exposed to either the CS-P cements or cement M were similar to that found in the control medium (Fig. 4A). We additionally performed the Live/Dead assay to evaluate the cytocompatibility of the CS-P cements, to visualize the living (green fluorescent)

Table 2  pH of α-medium and cement-immersed media

| Sample Label | α-medium | P    | D     | M     | CS-P1 | CS-P2 | CS-P3 |
|--------------|----------|------|-------|-------|-------|-------|-------|
| pH           | 7.6±0    | 7.7±0.3 | 11.2±0.3 | 8.6±0.2 | 11.0±0.2 | 10.0±0.4 | 8.9±0.2 |

Data are mean of three times measurements of each sample.

Fig. 2 Setting times for gypsum-containing calcium silicate cements.
A: Setting times for each cement in Table 1; note, CS-P cements were mixed with DW, water/powder ratio=0.5. B: Setting times for each cement in Table 1; note, CS-P cements were mixed with DW, water/powder ratio=0.4. Different lowercase letters (a–e) in the graph indicate significant difference. Data are mean±SD (n=3).

Fig. 3 Compressive strength of gypsum-containing calcium silicate cements.
Data are mean±SD (n=3). Different lowercase letters (a–e) in the graph indicate significant difference.
Fig. 4 Evaluation of the cytotoxicity and cell viability of cement-immersed media after 48 h in culture.
A: Evaluation of LDH activity leaked by damaged cells cultured in the media exposed experimental cements. Data are mean±SD (n=6). Different lowercase letters (a–c) in the graph indicate significant difference. B: Microscopic images of hDPSCs after treatment of the media exposed experimental cements with Live/Dead staining. (green color: live cells; red color: dead cells; scale bar: 200 µm)

Fig. 5 Evaluation of the cell growth and ALP activity of hDPSCs in cement-immersed media after 48 h and 7 days in culture respectively.
A: Data are mean±SD (n=6). Different lowercase letters (a–e) in the graph indicate significant difference. B: ALP activity staining images of hDPSCs after 7 days of culture in the five types of cement immersed media. (scale bar: 200 µm).

and dead cells (red fluorescent). As shown in Fig. 4B, many red spots were observed in the hDPSCs cultured in the medium exposed cement D. Several red spots were also observed in the cells cultured in the medium exposed cement P and M. On the other hand, the red spots observed in the cells cultured in the media exposed cement CS-Ps were few.

Evaluation of the proliferation of hDPSCs in cement-immersed media
The proliferation of hDPSCs was prominently inhibited in the medium exposed to cement D; this suppression was also observed in the media exposed to CS-P (0.4)-1, CS-P (0.4)-2, and cement M. The medium exposed to CS-P (0.4)-3 displayed only a slight suppression in the proliferation of hDPSCs (Fig. 5A).

ALP activity of hDPSCs in cement-immersed media
Figure 5B shows staining for ALP activity in hDPSCs cultured in five types of cement-immersed media for 7 days. Results of the CS-P3 group and cement M group, which showed the remarkable stainability, are presented. The increase in ALP activity in these two groups was more pronounced than that in the control and cement P groups; cement D group had sparse cells that were poorly stained.

DISCUSSION
In endodontic treatment, MTA and calcium hydroxide-based products, such as Dycal®, have been used to induce the formation of reparative dentin. The growth of this reparative dentin is hypothetically induced by alkali stimulation. Although calcium hydroxide-based products are strongly alkaline, they may also strongly irritate dental pulp and may trigger extensive necrosis. The medium exposed to cement D displayed strong alkalinity, on par with that of an unbuffered medium sample. Alternatively, MTA is a known bioactive material that has been demonstrated to reliably yield positive patient outcomes when used as pulp capping[6,7,15-17,30,31]; this positive attribute is corroborated by our observations. That is, no drastic change in pH between the media exposed to cement D and cement M was observed. Due to its physical properties, MTA only slowly releases calcium hydroxide during the gradual setting reaction. MTA has been used for various purposes, for example, during the sealing of furcal perforations[6,11,20], as a direct pulp capping agent[6,7,15-17], and as a retrograde filling material[6,19,20,32]. However, MTA exhibits certain disadvantages such as high cost, difficulty in handling[6], and long time required for setting[6,33,34].

To inexpensively synthesize calcium silicates, which are the major component of MTA, we experimentally synthesized calcium silicate from easily procured chemical regents. The XRD peaks associated with calcium silicates indicate that our synthesis did produce calcium silicates; thus, we show that it is possible to obtain calcium silicates via the calcination method used in this study. Several peaks associated with calcium oxide were also observed in the XRD pattern; these are assumed to have been produced through a side reaction.
involving one of the starting materials, with a possible reaction being the degradation of calcium carbonate into calcium oxide (CaCO$_3$→CaO+CO$_2$) beginning at ~700–800°C$^{[35]}$. During our experiments, test samples—calcium carbonate mixed with silicon dioxide—were sintered at 1,300°C in an electric furnace after being packed inside an alumina crucible. Under these conditions, the degradation of calcium carbonate into calcium oxide is expected. Any CaO remaining within our samples was detected by XRD. When the synthesized calcium silicates were mixed with DW, calcium hydroxide formation was observed; this is attributed to the following reaction:

$$\text{Ca}_2\text{SiO}_4+\text{H}_2\text{O} \rightarrow \text{CaSiO}_3+\text{Ca(OH)}_2,$$

$$\text{CaO}+\text{H}_2\text{O} \rightarrow \text{Ca(OH)}_2$$

However, we found that the hydration reaction (setting) of the synthesized calcium silicates is slow and that the mechanical strength of the resulting cured cement is low; therefore, they were considered to be unsuitable for clinical application. Due to these unacceptable properties, we prepared gypsum-containing calcium silicate cements because gypsum is a compound contained in MTA.$^{[23,34]}$ We anticipated that the addition of gypsum would shorten the setting time. Yet, it was necessary to evaluate the material properties and cyto-compatibility of this new cement. Therefore, the setting time and the compressive strength of the prepared gypsum-containing calcium silicate cements were investigated. The setting time was shorter for all gypsum-containing cements compared to that of MTA; however, the measured compressive strength was significantly lower than that of either MTA or Dycal. However, the compressive strength could be improved in a predictable and reproducible fashion by varying the amount of added gypsum. In addition, the setting time was slightly reduced for cements prepared with w/p=0.4 in comparison with those prepared with w/p=0.5. In particular, this effect was most pronounced in CS-P3 cements, which has a significantly higher gypsum content, suggesting that the magnitude of the effects caused by added gypsum is dose-dependent.

The biocompatibilities of the calcium silicate cements with dental pulp were also investigated using cultures of hDPSCs. Our results revealed that the growth of hDPSCs was significantly inhibited in media exposed to cement D (Dycal). Numerous studies have reported that Dycal causes irritation of the dental pulp exposed to cement D (Dycal). In particular, the elevated pH of media exposed to either CS-P1 or CS-P2, compared to the pH of the media exposed to MTA, can be attributed to the formation of calcium oxide during calcination. On the other hand, the inhibition of cellular proliferation was insignificant in the medium exposed to CS-P3 compared to that observed with other samples. The hDPSCs in the CS-P3 cement-immersed medium which did not inhibit cell proliferation showed increased ALP activity, similar to that observed in the MTA cement-immersed medium. Reportedly, in osteoblasts and chondrocytes, calcification begins after increased ALP activity$^{[36,37]}$. However, the cells used in this experiment were derived from pulp, and although calcification was not examined, CS-P3 cement was shown to possibly contribute to the calcification of hard tissues. Currently, further and more detailed studies, such as the examinations of the involvement in calcification using bone marrow-derived cells, are required, and these studies are ongoing.

Thus, we obtained a cement with high cell affinity by the addition of 40% (wt/wt) of gypsum hemihydrate to the sintered calcium silicate.

**CONCLUSIONS**

Gypsum-containing calcium silicate cements were synthesized at lab-scale from readily available chemical reagents. Within this limited study, our investigations led to the following conclusions:

1. Calcium silicates could easily be synthesized from commercial calcium carbonate and silicon dioxide reagents.
2. With the addition of gypsum hemihydrate to the calcium silicate cements, both a reduction in the setting time and improvement in mechanical strength could be achieved.
3. Our novel gypsum-containing calcium silicate cement show limited cytotoxicity when compared to the calcium hydroxide agent.
4. Experimental CS-P3 cement showed high advantages owing to biocompatibility that does not inhibit cell growth and potential application in hard tissue regeneration by increasing ALP activity, in addition to the mechanical properties of synthetic cements.

These results suggested that the newly developed calcium silicate material could be a promising pulp capping material.

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