博 士 論 文

Physio-Chemical and Bioactive Properties of Experimental Calcium Silicate-Based Cement Containing Phosphate Compounds

（リン化酸合物を含んだカルシウムシリケートセメントの物理化学的性質と生体活性特性について）

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Abstract

Objectives: This laboratory study aimed at assessing the solubility, pH, calcium ion release, setting time, and hydroxyapatite (HAp) precipitation bioactivity of various calcium silicate-based cement (CSC) modified with short-chain polyphosphate (polyPS) or phosphorylated pullulan (PPL).

Methods: Four study groups were tested: NEX-MTA (NMTA; GC, Japan), NMTA with phosphorylated pullulan (MTA-PPL), NMTA with poly-PS (MTA-polyPS), and Biodentine (BD; Septodont, France). Solubility was measured after 7 and 28 days at 37°C in deionized water. Calcium ion release and pH evaluation were performed after immersion in deionized water at 3 h, 24 h, 7 days, and 28 days using pH and Ca^{2+} meters. Setting times were determined with a Vicat apparatus, and bioactivity was evaluated through scanning electron microscopy/energy-dispersive X-ray (SEM/EDX) and Fourier transform infrared spectroscopy (FTIR) analysis 28 days in phosphate-buffered saline.

Results: NMTA showed the lowest solubility, while MTA-PPL exhibited the highest. NMTA had the most alkaline pH at 7 and 28 days, whereas MTA-polyPS had a lower pH than its control. BD released the most Ca^{2+}, with MTA-polyPS releasing the least. BD exhibited the shortest initial and final setting time (9.2 and 42 min, respectively), followed by MTA-polyPS, MTA-PPL, and NMTA. SEM/EDX and FTIR indicated that MTA-polyPS had the highest HAp formation, with Ca/P ratios of 1.7 (NMTA) and 1.79 (MTA-polyPS). MTA-PPL and BD showed no significant HAp formation, with Ca/P ratios of 3.93 and 34.9, respectively.

Significance: The incorporation of polyPS into CSC may enhance HAp formation and accelerate the

setting without altering the solubility properties.

Keywords: Mineral trioxide aggregate, Calcium silicate cement, Hydroxyapatite, Bioactivity, Endodontics

1. INTRODUCTION

Mineral trioxide aggregate (MTA) is a highly biocompatible Ca-silicate-based cement (CSC) that demonstrates positive clinical outcomes in endodontic procedures [1][2]. MTA was the first CSC used in endodontics, and since then, various types of MTA have been developed [3]. MTA is used as pulp capping as an alternative to $Ca(OH)_2$, as it induces dentin development and reduces pulp inflammation [4][5]. Moreover, MTA possesses several advantageous physical properties compared to $Ca(OH)_2$, such as improved sealing, reduced solubility, and greater structural stability [2]. Therefore, MTA has recently been defined as the new gold standard in several endodontic procedures [6]. However, MTA still presents several drawbacks, including challenging handling, extended setting time, and potential for discoloration of the crown [7][8]. Furthermore, its challenging handling properties and relatively poor adhesion to hard dental tissues has motivated researchers to continue the investigation of new-generation CSCs [9].

Indeed, a modern CSC known as Biodentine™ (Septodont, Saint-Maur des Fosses, France), which was introduced in 2009 to improve some of the disadvantages aforementioned [10]. Biodentine™ exhibited

a shorter setting time and significantly reduced discoloration compared to conventional MTA, due to the absence of bismuth oxide and the additive of $CaCl_2$ acting as an accelerator and regulated the setting reaction. [11][12]. However, Biodentine™ still presents challenges in handling, does not adhere well to hard dental tissues, and its long-term *in vivo* effectiveness as a pulp-capping treatment remains uncertain [9].

In a recent study, it has been proposed that MTA containing phosphorylated pullulan (PPL) does not present some of the typical issues of CSCs, particularly the lack of adhesion to hard dental tissues, along with the difficult handling properties [13]. Pullulan (PL) is a polymer derived from the fermentation of black yeast [14]. As a potential biomaterial, pullulan offers several advantages due to its non-toxic nature and the availability of OH^- groups within the pyranose rings for substitution with phosphate groups [15][16]. Therefore, phosphorylated pullulan (PPL) has been expected as a carrier for growth factors for bone tissue engineering, where its strong chemical bond that forms between the phosphate groups and hydroxyl group contributes to adhesion with hard tissue [17]. Some studies have already demonstrated that MTA containing PPL (MTA-PPL) exhibits favorable bioactivities, including an excellent healing response in pulp tissue and the ability to induce complete mineral tissue formation when used as a direct pulp capping material [9][18][19].

On the other hand, inorganic polyphosphate (polyP) is composed solely of a chain of phosphate groups connected by high-energy bonds. Perhaps interestingly, this compound is present in every organism and plays roles in a multitude of cellular processes, including phosphate storage, blood coagulation,

and pathogenicity [20]. PolyP has been identified in human osteoblasts and is known to have several beneficial effects on bone tissue [21]. It also positively influences apoptosis and the mineralization process [22]. Furthermore, PolyP promotes intracellular calcification, the maturation of bone-related immature cells, and contributes to bone tissue deposition by osteoblasts; it induces alkaline phosphatase (ALP) activity, as well as upregulates the gene expression of osteocalcin and osteopontin [23]. PolyP present enhanced antibacterial properties and dentin-inductive properties, depending on its molecular size (chain length). PolyP is classified into long-chain (polyPL), medium-chain (polyPM), and short-chain (polyPS). It has been advocated that polyPM enhances fibroblast growth factor (FGF) function, accelerates tissue regeneration and bone regeneration, and inhibits bone resorption by osteoclasts, whereas polyPL showed similar activity as polyPM but act as a more efficient inhibitor of bone resorption and inducible nitric oxide, NO, synthase (iNOS) expression [24]. Moreover, polyPS has been demonstrated to be highly effective in preventing staining of the tooth surface and suppressing its demineralization by binding to the tooth surface [25]. Therefore, polyPS has most potential to strongly adhere to hydroxyapatite (HAp) compared with the other polyP with different chain lengths [26].

This laboratory study aimed to evaluate the solubility, pH, calcium ion release, initial and final setting times, and HAp deposition bioactivity of various MTA cements doped with poly-PS or PPL compared to two commercial MTA and Biodentine™. The hypothesis being tested in this study was that the incorporation of polyPS or PPL in an MTA cement would enhance its physio-chemical and bioactive

properties.

2. MATERIALS AND METHODS

This laboratory study was approved by the Institutional Ethical Committee of Hokkaido University (2018-9). The present investigation was designed with four experimental groups which are depicted in Table 1.

2.1. Materials

The materials used in this study are also depicted in Table 1. NMTA, an MTA-based CS was used as a control material, and as the base material to formulate experimental MTA. Hence, several experimental MTA-PPL and MTA-polyPS were generated and used in this study. Biodentine™, a further popular CS-based material widely applied in various fields of dentistry, was also employed in this study as the control group.

Table 1: tested materials and their composition

Materials	Manufactures	Components
NEX-MTA™ (NMTA)	GC, Tokyo, Japan	Calcium oxide, Bismuth oxide, Silicon dioxide, Aluminum oxide
Mineral trioxide aggregate containing phosphorylated pullulan (MTA-PPL)	GC, Tokyo, Japan	Calcium oxide, Bismuth oxide, Silicon dioxide, Aluminum oxide, Phosphorylated pullulan
Phosphorylated Pullulan (PPL)	GC, Tokyo, Japan	Pure phosphorylated pullulan
Biodentin™ (BD)	Septodont, Saint Maur des	Powder: Tricalcium silicate, Dicalcium silicate,

	Fosses, France	Calcium carbonate, Zirconium dioxide, Ion oxide
		Liquid: Calcium chloride, Hydrosoluble polymer
Exclusive Polyphosphate Short chain™ (polyPS)	RegeneTiss, Tokyo, Japan	0.5 mol/L in H_2O

2.2. Specimen Preparation

The following materials were tested in the present investigation:

Group 1 - NMTA: NEX-MTA™, the powder was mixed at a water-to-powder ratio of 1:3 on a glass slab for 1 minute according to the manufacturer's instructions.

Group 2 - BD: Biodentine™; the powder was mixed with the liquid according to the manufacturer's instructions: open a Biodentine capsule and remove the white cap, use a pipette to dispense five drops of liquid into the capsule, close the capsule with the white cap, and triturate the mixture for 30 s at 4,000 rpm.

Group 3 - MTA-polyPS: An experimental MTA containing Exclusive Polyphosphate Short chain™ (MTA-polyPS); the experimental MTA was prepared by 0.5 mol/L polyPS to NMTA original composition instead of water. The powder was mixed with poly-PS solution the same technique used in Group 1.

Group 4 - MTA-PPL: the experimental MTA-PPL was prepared by adding 5 wt% PPL to NMTA. The powder was mixed at a water-to-powder ratio of 1:3 on a glass slab for 1 min in the same technique used in Group 1.

2.3. Solubility

To assess the solubility of the tested materials, silicone molds with an internal diameter of 7.75 mm and a thickness of 1.5 mm were utilized [27]. These were placed on a 1 mm thick glass plate and covered with a cellophane. After being filled with material, the molds were stored at 37°C and 95% relative humidity for a duration equal to three times the final setting time. After being removed from the molds, the specimens were weighed on a precision analytical balance with 0.0001 g accuracy (AUX220™; Shimadzu, Japan) - this weight was recorded as the initial (W_0). Subsequently, each specimen was individually placed in a cylindrical polystyrene container holding 7.50 mL of deionized water, ensuring that the material did not encounter the internal surface of the container. The recipients were sealed and stored at 37 °C. After 7 and 28 days, the specimens were taken out of the containers, rinsed with deionized water, and then placed in a desiccator for 24 h. Then, the specimens were weighed on the same balance and this weight was recorded as the final (W_f). The solubility value at 7 and 28 days corresponded to the percentage of weight variation at these times about the W_0, according to the formula:

$$\text{solubility} = (W_f - W_0) \times 100. \quad (1)$$

If any specimen showed signs of disintegration, it was replaced, and the test was repeated. Five specimens were used for each group, and the mean values and standard deviation (%) were calculated to determine the solubility value [28].

2.4. Alkalizing Activity (pH) and Calcium Ion Releasing Activity

The specimens for measuring pH and calcium-releasing activity were prepared and stored in deionized water in the same way as the solubility test. The pH values were recorded using a pH meter (Sension™+PH3; Hach Lange, UK), and Ca^{2+}-releasing activity was measured using a calcium ion meter (pH80+DHS; Dostmann Electronic, Germany) [29] at three times for each experiment: after 3 h, 24 h, 7 and 28 days. Before measuring, the pH meter was calibrated with solutions of known pH (4.0, 7.0 and 9.0). Before the immersion of the specimens, the pH of the deionized water was checked to avoid any interference. Five measurements were recorded for each group, and the mean values and standard deviation were calculated to obtain the pH value [28][30].

2.5. Initial and Final Setting Time

The setting time was determined using a Vicat apparatus (Contenco, São José da Lapa, MG, Brazil), as recommended by ANSI/ADA 57 [31]. The mixed cements were mixed and placed into silicone molds (10 mm in diameter and 2 mm in height). Five measurements were taken for each group. The Vicat needle was lowered and held in contact with the material's surface for 5 s to assess the setting properties. This procedure was repeated every 60 s, continuing until the indentation test made by the Vicat needle on the material's surface was no longer visible to the naked eye. The test was conducted three times for consistency, and the setting time was established based on the mean value obtained from these repetitions [32]. The initial setting time was measured at 1-minute intervals for the first 10

min. The point at which the needle could no longer penetrate to the full depth of 2 mm in the cement was designated as the initial setting time. The final setting time was then measured at 5-minute intervals from 10 to 30 min, followed by 1-hour intervals from 30 to 120 min. To accurately pinpoint the final setting time, measurements were again taken at 1-minute intervals as the final setting approached. The specimens were stored in a cabinet maintained at 37°C and 95% relative humidity during this latest setting steps. The moment when the needle left no visible impression on the specimen was recorded as the final setting time. To confirm that the setting was fully complete, two additional measurements were conducted after the final setting point was determined [33].

2.6. Scanning Electron Microscopy and Energy-Dispersive X-ray Analysis

The specimens (three specimens per group) were prepared in the same way described above and stored at 37 °C and 95% relative humidity. The ability of the materials to induce apatite precipitation bioactivity was performed by immersing the cement disks at 37 °C in 13 mL phosphate-buffered saline (PBS) for 0 days (baseline) and 28 days. The specimens from each group were mounted on aluminum stubs and coated with gold using a sputter coater (MED 010; Balzers, Balzer, Liechtenstein). They were then examined using an ultra-high-resolution analytical focused ion beam scanning electron microscope (FIB-SEM, Thermo Scientific Scios 2 DualBeam, Waltham, MA, USA) in the secondary electron mode. The FIB-SEM was equipped with an energy-dispersive X-ray spectroscope (EDX), which operated at 20 kV voltage. During the EDX analysis, each specimen was assessed in six different

random spot areas to determine the Ca/P ratio of the crystals formed [34].

2.7. Fourier Transform Infrared Analysis

The specimens (three specimens per group) were prepared as described in chapter 2.2 and stored at 37 °C and 95% relative humidity. These were evaluated via a Fourier-transform infrared (FTIR) micro-spectroscopic system equipped with an attenuated total reflection (ATR) device (Spectrum Two, Perkin Elmer, Madrid, Spain). The specimens were submitted to vibrational chemical analysis in triplicates and the spectra were recorded in the wavenumber range 3000–500 cm^{-1} and with 32 scans at 4 cm^{-1}. The FTIR peaks were analyzed after baseline (0 days) and 28 days in PBS immersion. The data were next processed via subtraction and normalization using the Spectrum 10™ software (Perkin Elmer) to identify the most characteristic inorganic compounds in the specimens.

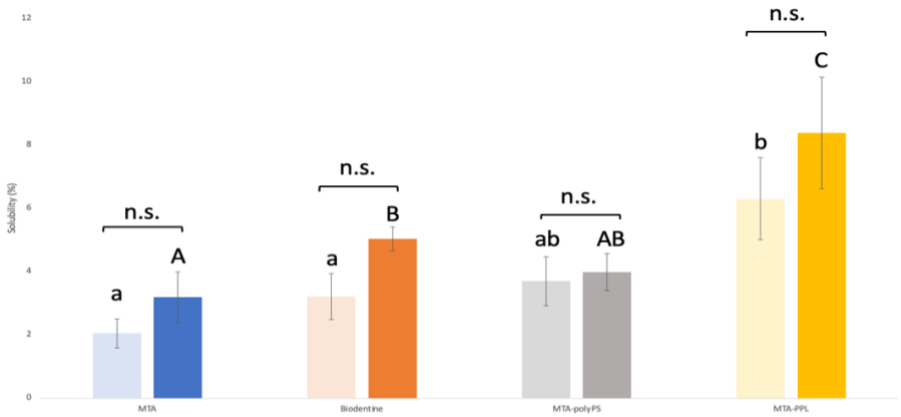
2.8. Statical analysis

The Steel-Dwass multiple comparison test was employed to compare data within the same measurement periods for solubility, pH, calcium and fluoride ion release, and setting time tests. In contrast, when comparing data across different measurement periods, Welch's T-test was applied for the solubility test, while the Steel-Dwass test was utilized for pH, calcium and fluoride ion release, and setting time tests. The P-value of less than 0.05 was regarded as statistically significant.

3. RESULTS

3.1. Solubility

The results of the solubility test are shown on Fig. 1. NMTA exhibited the lowest solubility at both 7 and 28 days. Whereas MTA-PPL exhibited the highest solubility at both 7 and 28 days. The solubility of BD was lower than MTA-polyPS at 7 days, but it became higher than MTA-polyPS at 28 days. Statistical analysis showed that the solubility of MTA-PPL had significantly higher differences ($P<0.05$) compared to NMTA and BD at 7 days. On the other hand, MTA-PPL had a significantly higher difference compared to the other groups at 28 days. Moreover, the solubility of BD was significantly higher than NMTA at 28 days. It can be said that MTA-PPL has significantly higher ($P<0.05$) solubility compared with the other groups. There were no significant differences between the solubilities of NMTA and MTA-polyPS. Moreover, there were also no significant differences ($P>0.05$) in solubility between the 7-day and 28-day groups in the same experimental material.



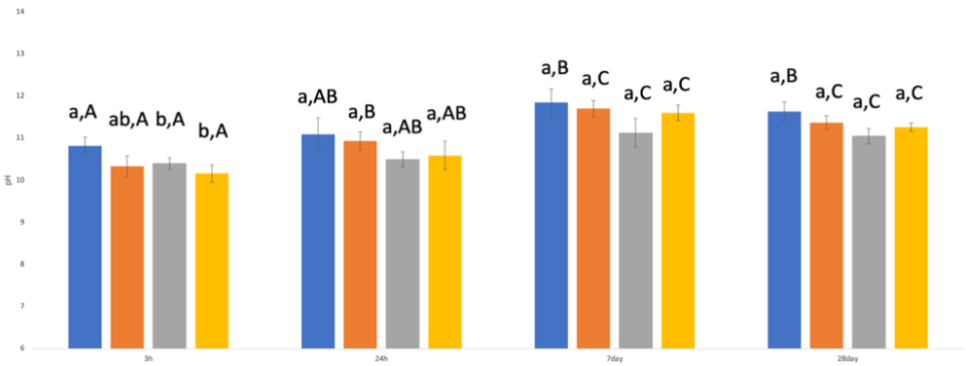
Solubility (%)	NMTA	Biodentine	MTA-polyPS	MTA-PPL
7 days	2.05 ± 0.46	3.21 ± 0.73	3.7 ± 0.77	6.31 ± 1.3
28 days	3.19 ± 0.8	5.04 ± 0.37	3.99 ± 0.59	8.39 ± 1.76

Figure 1: Average and standard division of solubility recorded for each set of specimens. Different lowercase letters indicate statistically significant differences in 7 days solubility. Different capital letters indicate statistically significant differences in 28 days solubility ($p < 0.05$). There were also no significant differences in the solubility between the 7-day and 28-day groups in the same experimental material ($p > 0.05$).

3.2. Alkalizing Activity (pH) and Calcium Ion Releasing Activity

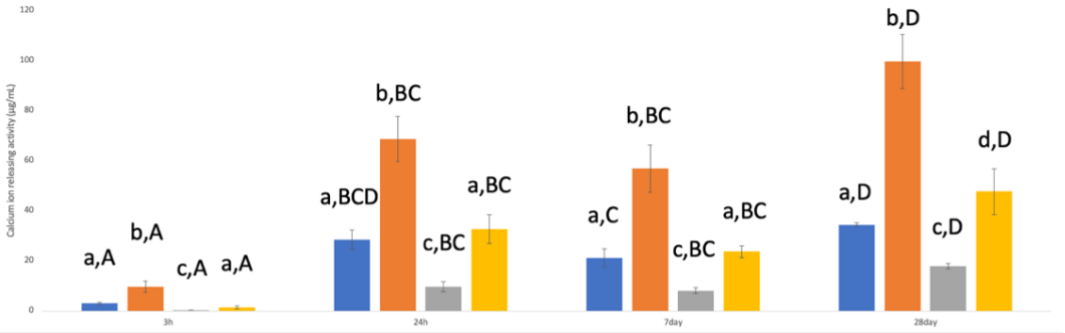
The result of alkalizing activity (pH) is shown in Fig. 2. NMTA exhibited the highest pH in all measurement periods ($P<0.05$). BD Group (Biodentine™) showed the second highest pH values, while MTA-polyPS exhibited relatively lower pH in all periods ($P<0.05$). Statistical analysis showed that MTA-polyPS and MTA-PPL exhibited a significantly lower pH ($P<0.05$) compared to NMTA at 3h. However, in the other measurement periods, there were no significant differences ($P>0.05$). Therefore, MTA-polyPS and MTA-PPL showed significantly ($P<0.05$) lower pH at the initial period, but they became gradually comparable with the other specimens ($P>0.05$).

The results of Ca^{2+}-releasing activity are shown in Figure 3. BD Group showed the highest ($P<0.05$) release of Ca^{2+} in all measurement periods. Whereas MTA-polyPS showed the lowest ($P>0.05$) calcium release in all measurement periods. The calcium-releasing activities of NMTA and MTA-PPL were similar. Statistical analysis showed that there were no differences ($P>0.05$) between the calcium releasing activity between NMTA and MTA-PPL in 3 h, 24 h, and 7 days. However, the calcium-releasing activity of MTA-PPL was significantly higher ($P<0.05$) than NMTA at 28 days. NMTA and MTA-PPL had significantly higher calcium ion releasing activities than MTA-polyPS in all observation periods ($P<0.05$).



pH	3 hours	24 hours	7 days	28 days
NMTA	10.83 ± 0.21	11.10 ± 0.39	11.86 ± 0.32	11.64 ± 0.23
Biodentine	10.34 ± 0.25	10.95 ± 0.21	11.71 ± 0.19	11.38 ± 0.16
MTA-polyPS	10.41 ± 0.13	10.51 ± 0.18	11.14 ± 0.34	11.06 ± 0.18
MTA-PPL	10.17 ± 0.20	10.60 ± 0.34	11.61 ± 0.18	11.27 ± 0.10

Figure 2: Average and standard division of pH recorded for each set of specimens. Different lowercase letters indicate statistically significant differences between different experimental materials in the same measuring period ($p<0.05$). Different capital letters indicate statistically significant differences between the same experimental material in the different measuring periods ($p<0.05$).



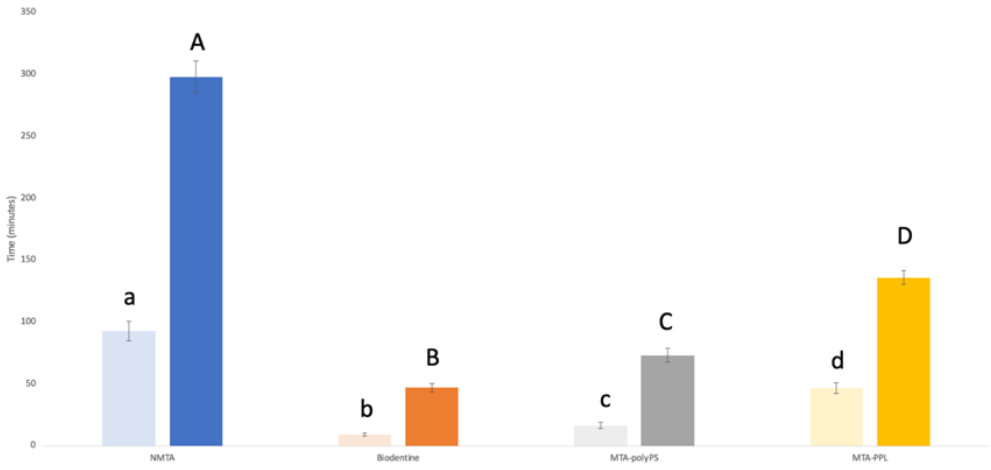
Calcium ion releasing activity (μg/mL)	3 hours	24 hours	7 days	28 days
NMTA	3.01 ± 0.45	28.48 ± 3.82	21.16 ± 3.54	34.43 ± 0.67
Biodentine	9.63 ± 2.25	68.73 ± 9.06	56.82 ± 9.33	99.62 ± 10.87
MTA-polyPS	0.25 ± 0.10	9.58 ± 2.02	8.07 ± 1.13	17.89 ± 0.99
MTA-PPL	1.45 ± 0.63	32.58 ± 5.76	23.64 ± 2.39	47.63 ± 9.25

Figure 3: Average and standard division of calcium ion-releasing activity recorded for each set of specimens. Different lowercase letters indicate statistically significant differences between different experimental materials in the same measuring period ($p<0.05$). Different capital letters indicate statistically significant differences between the same experimental material in the different measuring periods ($p<0.05$).

3.3. Initial and Final Setting Times

The results of the initial and final setting times are shown below in Fig. 4. Overall, the initial and final

setting times of the specimens followed this order: NMTA had the longest setting time, followed by MTA-PPL, MTA-polyPS, and Biodentine™ with the shortest setting time. NMTA showed the longest setting time compared with the other groups (P<0.05). BD Group showed the shortest setting times (P<0.05) than all the other tested materials. MTA-polyPS exhibited significantly shorter setting times compared with NMTA and MTA-PPL (P<0.05). The setting time of MTA-PPL was significantly shorter than NMTA (P<0.05).



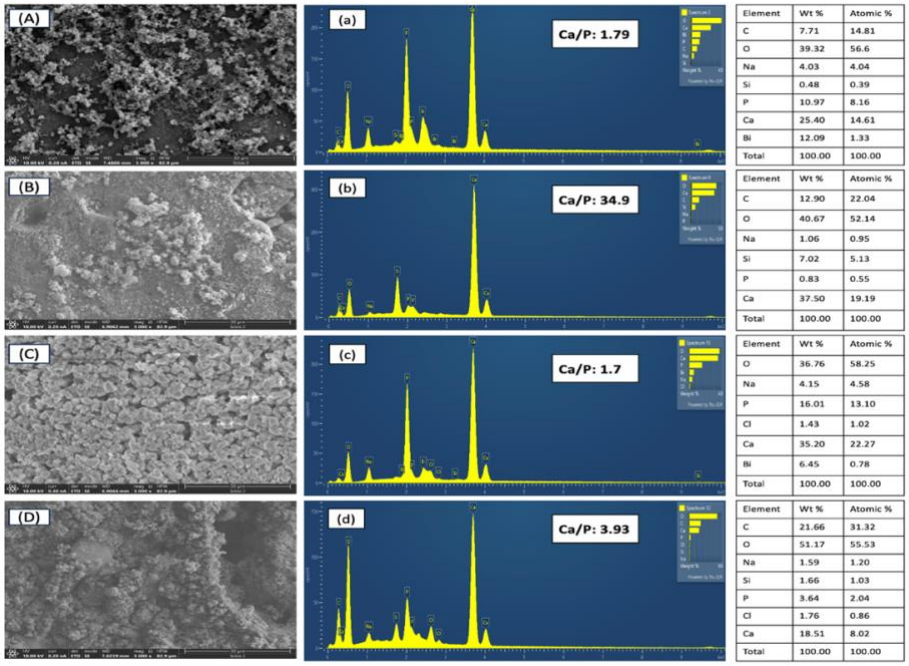
Setting times (min)	NMTA	Biodentine	MTA-polyPS	MTA-PPL
Initial setting time	93 ± 7.8	9.2 ± 1.2	16.4 ± 2.6	46.6 ± 4.2
Final setting time	298.2 ± 12.7	47 ± 3.5	73.2 ± 5.5	136 ± 5.5

Figure 4: Average and standard division of initial and final setting time recorded for each set of specimens. Different lowercase letters indicate statistically significant differences in initial setting time (p <0.05). Different capital letters indicate statistically significant differences in final setting time (p <0.05).

3.4. Scanning Electron Microscopy and Energy-Dispersive X-ray Analysis

The results of SEM of the surfaces' tested materials (×5000) and SEM/EDX analysis with EDX Ca/P ratio are shown in Fig. 5. Additionally, SEM surface images with the high magnification (×10000) are

shown in Fig. 6. In SEM surface images at high magnification, (Fig. 6A) and (Fig. 6C) images exhibited crystal deposition. On the other hand, (Fig. 6B) and (Fig. 6D) images did not exhibit it. EDX Ca/P ratios of NMTA, BD, MTA-polyPS, and MTA-PPL were 1.79, 34.9, 1.7, and 3.93, respectively.



Element	Wt %	Atomic %
C	7.71	14.81
O	39.32	56.6
Na	4.03	4.04
Si	0.48	0.39
P	10.97	8.16
Ca	25.40	14.61
Bi	12.09	1.33
Total	100.00	100.00

Element	Wt %	Atomic %
C	12.90	22.04
O	40.67	52.14
Na	1.06	0.95
Si	7.02	5.13
P	0.83	0.55
Ca	37.50	19.19
Total	100.00	100.00

Element	Wt %	Atomic %
O	36.76	58.25
Na	4.15	4.58
P	16.01	13.10
Cl	1.43	1.02
Ca	35.20	22.27
Bi	6.45	0.78
Total	100.00	100.00

Element	Wt %	Atomic %
C	21.66	31.32
O	51.17	55.53
Na	1.59	1.20
Si	1.66	1.03
P	3.64	2.04
Cl	1.76	0.86
Ca	18.51	8.02
Total	100.00	100.00

Figure 5: The results of SEM for each specimen of low magnification (×5000) where (A) NMTA, (B) Biodentine™ (C) MTA-polyPS, (D) MTA-PPL), and EDX analysis with Ca/P ratio: (a) for (A), (b) for (B), (c) for (C), and (d) for (D).

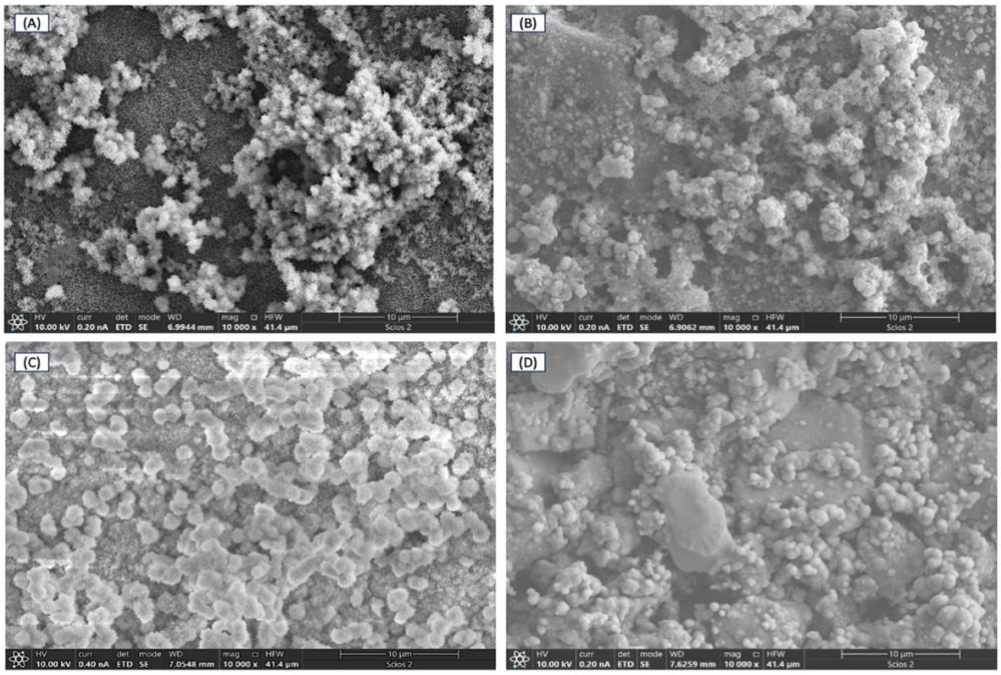


Figure 6: The results of scanning electron microscopy for each specimen at high magnification showing mineral deposition (×10000; (A) NMTA, (B) Biodentine (C) MTA-polyPS, (D) MTA-PPL).

3.5. Fourier Transform Infrared Analysis

The results of FTIR analysis of each specimen, time 0 (baseline) and 28 days in PBS immersion, are presented in Fig. 7. At time 0 (baseline), only MTA-polyPS exhibited PO stretching of hydroxyapatite peaks at wavenumbers 560.89 cm^{-1}, 599.60 cm^{-1}, and 1038.23 cm^{-1} in both sections [A] and [B]. On the other hand, NMTA exhibited PO stretching of hydroxyapatite peaks at 556.3 cm^{-1} and 1053.9 cm^{-1}, and MTA-polyPS exhibited peaks at 583.1 cm^{-1}, 599.60 cm^{-1}, and 1038.23 cm^{-1} in both sections [A] and [B] in 28 days. The stronger peaks of MTA-polyPS in 28 days were confirmed than time 0. The peaks of NMTA were observed relatively weaker in 28 days.

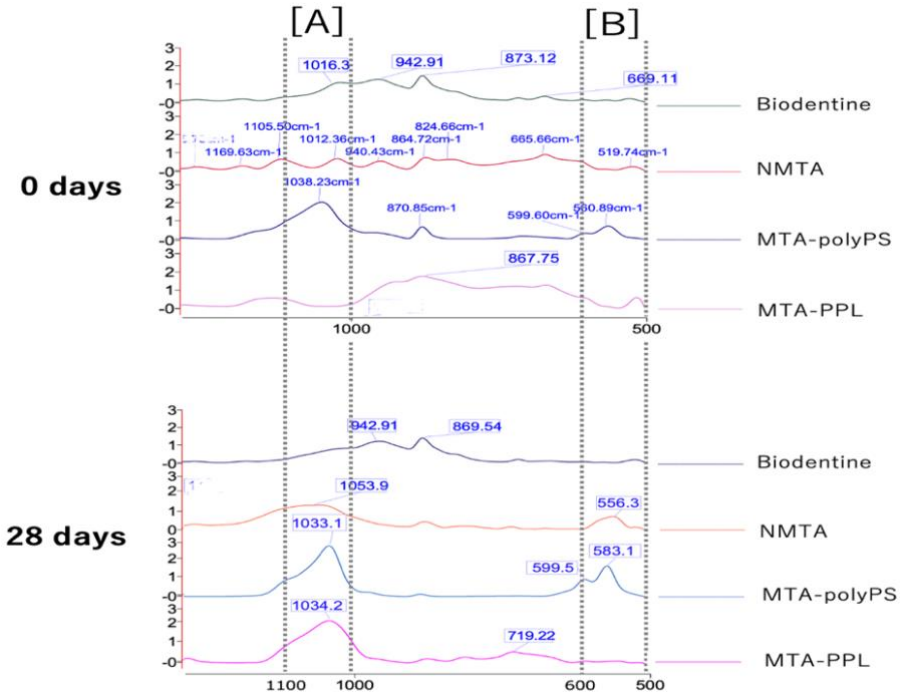


Figure 7: The results of FTIR analysis of each specimen, time 0, and time aging. The spectra from top to bottom of the figure are BD, NMTA, MTA-polyPS, and MTA-PPL, respectively. In the wavelength range between 1000 cm^{-1} to 1100 cm^{-1} in section [A], and the spectra range between 500 cm^{-1} to 600 cm^{-1} in section [B], it is possible to observe the most representative peaks of apatite deposition.

4. DISCUSSION

The hypothesis that the incorporation of polyPS or PPL in an MTA cement would enhance its physio-chemical and bioactive properties must be only partially accepted. The reason for such a conclusion is related to the results obtained in the different laboratory tests performed in this study. Indeed, according to the solubility test, MTA-PPL exhibited the highest solubility. This result may be correlated to the fact that pullulan is an easily water-soluble material due to its hydrophilic phosphate groups. It therefore may have absorbed excessive water, causing an alteration of the setting reaction as well as great porosity [35]. Biodentine™ exhibited higher solubility than NMTA; this result is in accordance with a previous study that showed higher solubility in Biodentine™ than conventional MTA [36]. Considering the requirements of the ISO 6876:2001 standard [37], which specifies that a set sealer should be less soluble than 3% in distilled water, Biodentine™ and MTA-PPL, which have comparatively higher solubility, may not be suitable for use as sealers [38][39]. Alkaline pH is important in biocompatibility, antibacterial activity, and osteogenic potential [40]. NMTA exhibited the highest pH at all tested periods. MTA-polyPS and MTA-PPL, which were based on NMTA incorporated with phosphate groups, exhibited significantly lower pH than NMTA at 3 h, but there was no significant difference between the experimental groups and NMTA at 24 h, 7 days, or 28 days. Therefore, it can be affirmed that the effect of the polyPS and PPL on the pH of NMTA is only limited in the initial stage.

On the other hand, MTA-polyPS exhibited significantly shorter initial and final setting times than

NMTA. This was probably due to polyPS influencing the tricalcium silicate's and dicalcium silicate's hydration reaction, enhancing the precipitation of insoluble Ca-silicate hydrate and $Ca(OH)_2$ crystals [41]. The pH of MTA-polyPS was significantly lower than NMTA at 3 h. In other words, it can be said that poly-PS consumes the $Ca(OH)_2$ in the initial stage of the hydration reaction of calcium silicates because the lower amount of calcium hydroxide produced by the hydration reaction contributes to the lower pH of MTA [42]. Considering the Ca^{2+} release of MTA-polyPS was significantly shorter than that of NMTA at 3 h, phosphate ion from poly-PS might react with the calcium hydroxide to form amorphous phosphate, which eventually yields hydroxyapatite (HAp) [43]. Indeed, FTIR analysis at the baseline of MTA-polyPS confirmed the formation of HAp. Furthermore, MTA-PPL also exhibited significantly shorter initial and final setting time with a significantly lower pH than NMTA at 3 h, but there were no significant differences in Ca^{2+} release between NMTA and MTA-PPL at 3 h. Moreover, FTIR analysis at the baseline showed MTA-PPL did not form any HAp. On the other hand, the incorporation of natural modifier polymers such as fucoidan into MTA was reported to improve the setting time as a result of the acceleration of the hydration process by its cross-linking structure [44]. It can be concluded that MTA-PPL might show a shorter setting time than NMTA mainly because of its polymer structure. Biodentine™ showed the significantly shortest setting time; the the absence of Bi_2O_3 and in particular the presence of $CaCl_2$ and other accelerators contained in the liquid may have acted as an accelerator and regulated the setting reaction, [11][45]. On the other hand, Biodentine™ exhibited the significantly highest calcium ion-releasing activity. This

result also coincides with a previous study that showed Biodentine™ exhibited significantly higher calcium ion-releasing activity compared with conventional MTA [46].

Concerning the bioactivity of forming HAp, SEM/EDX analysis showed that EDX Ca/P ratio of NMTA and MTA-polyPS were 1.79 and 1.70, respectively. Considering that the EDX Ca/P ratio of HAp is within the range of around 1.5 to 1.8 [47][48], it can be said that NMTA and MTA-polyPS have the potential to form HAp. This result coincides with the apatite-like crystallization of NMTA and MTA-polyPS observed in high-magnification SEM surface images. Moreover, FTIR analysis also supported this result. In the FTIR analysis of NMTA and MTA-polyPS after aging in PBS immersion, PO stretching of HAp peaks at 500 cm^{-1} to 600 cm^{-1} and 1000 cm^{-1} to 1100 cm^{-1} were confirmed [49]. MTA-polyPS might be a more promising HAp-forming material because the HAp peaks of MTA-polyPS were confirmed more clearly than those of NMTA. At baseline, only MTA-polyPS exhibited HAp peaks in both sections [A] and [B]. This could mean MTA-polyPS has the potential to form HAp at an early stage compared with NMTA (Figure7). This result supports the idea that conventional MTA, such as NMTA, has the potential to form HAp when immersed in PBS [49][50]. On the other hand, in this limited study, Biodentine™ and MTA-PPL did not form HAp. Some other studies have shown that Biodentine™ possesses the potential to induce the crystallization of minerals composed of calcium and phosphates subsequent prolonged PBS immersion [51][52]. However, it could be said that such a crystallization is only a kind of amorphous Ca-phosphate, not HAp. The reason MTA-PPL did not form HAp is uncertain; however, considering MTA-PPL was based on NMTA, it might be said that

PPL interfered with the precipitation of Hap crystals when incorporated in NMTA.

Indeed, several studies have shown that MTA-PPL displays positive bioactivities, such as promoting excellent healing responses in the pulp tissue and facilitating complete mineral tissue formation, making it effective as a direct pulp capping material [9][18][19]. MTA-PPL showed comparable calcium ion-releasing activity and significantly shorter setting time compared with NMTA, but the higher solubility of MTA-PPL should be considered to apply it in the clinic, especially as a sealer, because the stability of the material may be the most critical factor from a long-term perspective [38][39]. On the other hand, MTA-polyPS showed significantly shorter setting time than MTA-PPL, comparable solubility with NMTA, and excellent bioactivity in forming HAp. This bioactivity in forming HAp of MTA is essential for increasing the sealing ability between the material and the dentin wall; such a characteristic may also influence the regeneration and remineralization of teeth [53].

Furthermore, it is noteworthy that the current study suggested that Ca^{2+} release was not essential for HAp formation. The calcium ion release activity of MTA-polyPS was significantly lower compared to the other experimental groups. While Biodentine™ exhibited much higher calcium ion release, it resulted in almost no HAp formation. Indeed, some studies have concluded that lower calcium ion release in calcium silicate-based cements correlates with reduced HAp formation [54][55]; however, Ca^{2+}release may be primarily associated with dentin formation or the activation of dental pulp stem cells involved in the formation of reparative dentin matrix [56][57]. Additionally, given that apatite-like crystalline deposits from CSC were observed with relatively lower Ca/P ratios in PBS immersion,

it suggests that phosphate ions may play a crucial role in HAp formation [58]. In addition, previous studies have already shown that phosphate is essential to form HAp in human bone [59][60]. Therefore, it can be suggested that phosphate ions may be a more critical factor in the formation of HAp or apatite-like crystalline deposits than calcium ions in endodontic procedures. Future *in vivo* studies on MTA-polyPS are anticipated to shed further light on this. On the other hand, further studies should be conducted to evaluate the bioactivity in forming hydroxyapatite of experimental materials under more clinical conditions, such as immersion in simulated body fluid, and to investigate how MTA-polyPS is involved in the formation of hydroxyapatite using methods such as gene expression, protein, and functional analysis.

5. CONCLUSIONS

Doping MTAs with additives containing phosphate groups, such as polyPS, might reduce the setting time, and increase the bioactivity in terms of hydroxyapatite deposition. Indeed, phosphate ions play a more crucial role than calcium ions in the formation of hydroxyapatite or apatite-like crystalline deposits.

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