Article

In Vitro Screening of the Apatite-Forming Ability, Biointeractivity and Physical Properties of a Tricalcium Silicate Material for Endodontics and Restorative Dentistry

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Abstract: *Aim*: Calcium silicate-based materials are hydraulic self-setting materials with physico-chemical properties suitable for endodontic surgery and good biological/clinical outcomes. The study aim was to evaluate the bio-properties (biointeractivity and apatite-forming ability) and selected physical properties (porosity, water sorption, solubility, and setting time) of Biodentine, a tricalcium silicate material for endodontics and restorative dentistry, compared to that of ProRoot MTA (Mineral Trioxide Aggregate) as gold standard material. *Methods*: Biodentine and ProRoot MTA pastes were prepared and analyzed for calcium release and alkalinizing activity (3 h–28 days), setting time, water sorption, porosity, solubility, surface microstructure and composition, and apatite-forming ability in simulated body fluid. *Results*: Biodentine showed higher calcium release, alkalinizing activity, and solubility but higher open and apparent porosity, water sorption,

and a markedly shorter setting time. Calcium phosphate (CaP) deposits were noted on material surfaces after short ageing times. A CaP coating composed of spherulites was detected after 28 days. The thickness, continuity, and Ca/P ratio of the coating differed markedly between the materials. Biodentine showed a coating composed by denser but smaller spherulites, while ProRoot MTA showed large but less dense aggregates of spherulitic deposits. ***Conclusions*:** Biodentine showed a pronounced ability to release calcium and extended alkalinizing activity interlinked with its noticeable porosity, water sorption, and solubility: open porosities provide a broad wet biointeractive surface for the release of the calcium and hydroxyl ions involved in the formation of a CaP mineral. Biodentine is a biointeractive tricalcium silicate material with interesting chemical-physical properties and represents a fast-setting alternative to the conventional calcium silicate MTA-like cements.

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1. Introduction

Calcium silicate-based materials, commonly known in dentistry as MTA (mineral trioxide aggregate), belong to a category of hydraulic self-setting cements mainly composed of di- and tricalcium silicates. They are hydrophilic radiopaque materials, which form a sticky self-setting calcium-silicate-hydrate (CSH) gel [1,2].

The father of the family of calcium silicate cements was innovatively introduced as tooth-filling material in 1995 by Torabinejad and White [3] as grey ProRoot® MTA (PROROOT® trademark registration 2000 by Dentsply International Inc., York, PA, USA; MTA® trademark registration in 1996 by Tulsa Dental Products and now abandoned) composed of grey Portland cement containing bismuth oxide as a radiopacifier [4].

The tooth-colored version, named white ProRoot MTA, was subsequently introduced in 2004 (trademark of ProRoot® registered in 2000 by Dentsply International Inc., York, PA, USA) by Primus [5] as an iron-free white Portland cement.

ProRoot MTA was initially proposed for root therapies (retrograde surgical filling/root-end sealing and root perforation repair/root and furcation perforations, internal/external resorptions) and revolutionized operative dentistry. ProRoot MTA has recently been considered for pulp therapy (in pulp capping, in deep cervical or radicular lesions, and for apexification and apexogenesis) [6,7] in view of its special chemical-physical and biological properties [1,8,9,10–18], as shown by its ability to release Ca^{+2} and OH^- ions in the surrounding environment [18–20] and to form apatite (bioactivity) [2,20–22] when in contact with (body) fluids.

Calcium silicate-based cements seem to have intrinsic properties suitable for their clinical use (apicoectomy, root perforation, apexification, and apical barrier) [3,23], such as good sealing [8,23,24] correlated to expansion [25,26], and the ability to set in the presence of fluids, bioactivity, the release

of ions acting as epigenetic signals [13,16,18–20], and good biological properties. Therefore, new calcium silicate MTA-like cements have recently been introduced.

Biodentine® is a calcium silicate-based material introduced in 2010 by Gilles and Olivier [27] with formulations quite different from ProRoot MTA but proposed for similar clinical applications in pulp capping treatments and as material for dentin restoration (material data sheet; [28]). Biodentine is composed of a tricalcium silicate powder packaged in capsules (0.7 g) to be mixed with a liquid phase (0.18 mL) containing calcium chloride. Biodentine proved able to promote the formation of reparative dentin [28] and positive results on odontoblasts when used in direct pulp capping [29–31].

The aim of the present study was to evaluate the bio-properties (biointeractivity and apatite-forming ability) and selected physical properties (porosity, water sorption, solubility, and setting time) of a recent calcium silicate-based material (Biodentine, Septodont, Saint-Maur-des-Fossés, France). ProRoot MTA represented the comparison material as a conventional long-standing progenitor of calcium silicate materials. The null hypothesis was that there is no significant difference between Biodentine and control.

2. Materials and Methods

2.1. Materials

Biodentine (Septodont, Saint-Maur-des-Fossés, France; batch number B01767) and ProRoot MTA (Dentsply Tulsa, Johnson City, TN, USA; batch number 09003850) were examined.

Biodentine is composed of tri-calcium silicate powder radiopacified with zirconium oxide and aqueous solution containing calcium chloride with excipients (patent Gilles R & Olivier M, 2011). The trademark BIODENTINE® was registered (serial no. 77509635, registration no. 3713946) as a pharmaceutical preparation in 2008 by Schiller, Henri Numa Marcel (Paris, France).

ProRoot MTA is composed of calcium silicate powder (white Portland cement) radiopacified with bismuth oxide and water (patent Primus, 2004). The trademark PROROOT® was registered (serial n. 75896452, registration no. 2417556) as a dental compound used in restorative and endodontic procedures in 2000 by Dentsply International Inc. (York, PA, USA).

The materials were prepared following the manufacturer's directions. The liquid to powder ratio (L/P) was 0.26 for Biodentine and 0.31 for ProRoot MTA.

2.2. Biointeractivity: Alkalizing Activity (pH of Soaking Water) and Calcium Release

The materials were prepared according to the manufacturer's instructions. The fresh material pastes were placed into PVC molds (8.0 ± 0.1 mm diameter and 1.6 ± 0.1 mm thickness).

Material disks ($n = 8$ for each material) were immediately immersed in 10 mL of deionized water (pH 6.8) in polypropylene sealed containers and stored at 37 °C. The soaking water was collected and replaced at six endpoints (3 and 24 h and 3, 7, 14, and 28 days). The collected water was analyzed for pH and Ca by a potentiometric method under magnetic stirring at room temperature (24 °C).

The pH was measured using a selective temperature-compensated electrode (Sen Tix Sur WTW, Weilheim, Germany) connected to a multi-parameter laboratory meter (inoLab 750 WTW, Weilheim, Germany) previously calibrated with standard solutions.

The amount of calcium ions was measured using a calcium probe (Calcium ion electrode, Eutech instruments Pte Ltd, Singapore, Singapore) after addition of 0.200 mL (2%) of ionic strength adjuster (ISA, 4 moL/L KCl, WTW, Weilheim, Germany) [18,20]. Cumulative calcium release was calculated separately for each of the eight samples of material by adding up the amounts released at the six different endpoints. Then the mean and standard deviation were calculated.

2.3. Physical Properties: Porosity, Water Sorption, Solubility, and Setting Time

Material disks ($n = 8$ for each material) were prepared following the manufacturer's directions. The material pastes were placed in the molds and allowed to set (at 37 °C and 99% relative humidity) for a period equal to 70% of the final setting time (*i.e.*, a period 50% longer than the time stated by the manufacturer, according to ISO 6876 clause 7.7.2. Dental root canal sealing materials), that is, 9 min for Biodentine and 250 min for ProRoot MTA and then removed from the molds.

Each sample was weighed to determine the initial mass (I) and immediately immersed vertically in 20 mL of distilled water and placed at 37 °C and the mass while suspended in water (S) was determined. After 24 h immersion the specimens were removed from the water and the excess water from the surface from each sample was removed using a moistened filter paper (20 mL of distilled water dropped on a 9 cm wide 12.5 cm long glass plate covered by a filter paper) and the saturated mass (M) was recorded. Finally, the samples were dried at 37 °C until the weight was stable and the final dry mass (D) was measured. Each weight measurement was repeated three times using an analytical balance (Bel Engineering series M, Monza, Italy) and determined to the nearest 0.001 g.

The *volume of open pores* V_{OP} ($V_{OP} = M - D$), the *volume of the impervious portion* V_{IP} ($V_{IP} = D - S$), the *exterior volume V* ($V = M - S$) and the *apparent porosity P* ($P = [(M - D)/V] \times 100$) were calculated in cm^3 or in percentage, following Archimedes' principle (and according to ASTM C373-88).

The *water sorption A* ($A = [(M - D)/D] \times 100$) and the *solubility S* ($S = [(I - D)/D] \times 100$) were calculated as percentage of the original weight [18]. ISO 4049 (polymer-based restorative materials) was used as both calcium silicate cements are hydraulic self-curing materials and their polymerization is chemically activated by the water in the mixing liquid.

To evaluate the *setting time*, each freshly mixed cement paste was placed in a mold measuring 10 mm in diameter and 2 mm in thickness and then stored at 37 °C and 95% ± 5% relative humidity. The final setting time was registered when no indentation was caused by a needle weighing 453.6 g with a tip diameter of 1.06 mm.

2.4. Surface Characterization

Freshly prepared materials were immediately immersed vertically in 20 mL of HBSS (Hank's Balanced Salt Solution, Lonza Walkersville, Inc., Walkersville, MD, USA) used as a simulated body fluid and stored at 37 °C for 1, 7, and 28 days [18,20,22,32]. The HBSS was renewed weekly.

The surface of each damp sample was examined in wet conditions without any previous preparation [2] using an environmental scanning electron microscope (ESEM, Zeiss EVO 50; Carl Zeiss, Oberkochen, Germany) connected to a secondary electron detector for energy dispersive *X*-ray analysis (EDX; Oxford Instruments, Abingdon, UK) with computer-controlled software (Inca Energy Version 18, Oxford Instruments, Abingdon, UK), at low vacuum (100 Pascal), accelerating

voltage of 20 kV, working distance 8.5 mm, 0.5 wt% detection level, 133 eV resolution, amplification time 100 microseconds, measuring time: 600 s for element mapping and 60 s for spectra.

Elemental X-ray microanalysis (EDX) provided micro-chemical spectra, element mapping, and semi‑quantitative compositional tables (weight % and atomic % of the elemental composition). The Ca/P ratio was calculated from the atomic data obtained.

2.5. *Statistical Analysis*

The normally distributed data were analyzed by parametric test (Sigma Stat, San Jose, CA, USA) using two-way ANOVA followed by RM Student-Newman-Keuls test (statistically significant difference for $p < 0.05$). In the tables, small letters represent statistically significant differences in the same line (*i.e.*, between the materials) and capital letters indicate statistical significance in the same column (*i.e.*, among the times).

3. Results

3.1. *Biointeractivity: Alkalizing Activity (pH of Soaking Water) and Calcium Release*

Both materials induced alkalization of the soaking water that decreased with time but was still present at 28 days (Table 1A). They alkalized the soaking water to pH 11−11.5 at short times (until three days) then decreased, and after 14 days the pH was ÷ 8−9.

Table 1. Results of alkalinizing activity (pH of soaking water) (**A**), calcium released (ppm) (**B**), porosity (**C**), setting time, water sorption and solubility (**D**) of Biodentine and ProRoot MTA (statistical analysis by two-way ANOVA with RM Student-Newman-Keuls test, $p < 0.05$). Small letters represent statistically significant differences in the same line (namely between the materials) while capital letters indicate differences in the same column (that is among the test times).

(**A**)

	pH of soaking water	
	Biodentine	**ProRoot MTA**
3 h	11.62 ± 0.13 [A a]	10.89 ± 0.67 [A b]
1 day	11.65 ± 0.53 [A a]	10.52 ± 0.60 [A b]
3 days	10.90 ± 0.59 [B a]	9.09 ± 0.40 [B b]
7 days	9.19 ± 0.59 [C a]	8.87 ± 0.14 [B a]
14 days	9.56 ± 0.27 [C a]	8.33 ± 1.15 [C b]
28 days	9.48 ± 0.74 [C a]	7.88 ± 0.46 [C b]

(**B**)

	Calcium released (ppm)	
	Biodentine	**ProRoot MTA**
0 - 3 h	$92.83 \pm 12.73^{\text{A a}}$	$24.48 \pm 4.62^{\text{A b}}$
3 h - 1 d	$17.13 \pm 4.82^{\text{B a}}$	$37.63 \pm 13.80^{\text{A b}}$
1 - 3 d	$18.76 \pm 8.38^{\text{B a}}$	$17.40 \pm 4.5^{\text{A a}}$
3 - 7 d	$43.58 \pm 3.03^{\text{C a}}$	$16.50 \pm 6.19^{\text{A b}}$
7 - 14 d	$28.85 \pm 9.73^{\text{B a}}$	$30.00 \pm 7.30^{\text{A a}}$
14 - 28 d	$42.4 \pm 4.37^{\text{C a}}$	$20.52 \pm 6.05^{\text{A b}}$

(**C**)

	Porosity	
	Biodentine	**ProRoot MTA**
Volume of Open Pores (Vop, cm^3)	$0.02324 \pm 0.0010^{\text{a}}$	$0.0312 \pm 0.0025^{\text{b}}$
Volume of Impervious Portion (Vip, cm^3)	$0.0778 \pm 0.0033^{\text{a}}$	$0.0755 \pm 0.0054^{\text{a}}$
Exterior Volume (V, cm^3)	$0.1010 \pm 0.0050^{\text{a}}$	$0.1067 \pm 0.0076^{\text{a}}$
Apparent Porosity (Vop/V, %)	$22.98 \pm 0.23^{\text{a}}$	$29.24 \pm 0.84^{\text{b}}$

(**D**)

	Physical properties	
	Biodentine	**ProRoot MTA**
Setting time (min)	$12 \pm 1^{\text{a}}$	$170 \pm 3^{\text{b}}$
Water sorption (%)	$12.64 \pm 0.15^{\text{a}}$	$14.55 \pm 0.44^{\text{a}}$
Solubility (%)	$11.93 \pm 0.43^{\text{a}}$	$10.70 \pm 0.33^{\text{a}}$

Both materials released Ca and the release decreased with the soaking time (Table 1B, Figure 1).

Biodentine showed a very high Ca release at both short (3 h) and long times (28 days) whilst the amount of Ca leached by ProRoot MTA was not marked but constant (no statistically significant changes over time).

Figure 1. Cumulative calcium released in soaking water.

Cumulative calcium released (ppm)
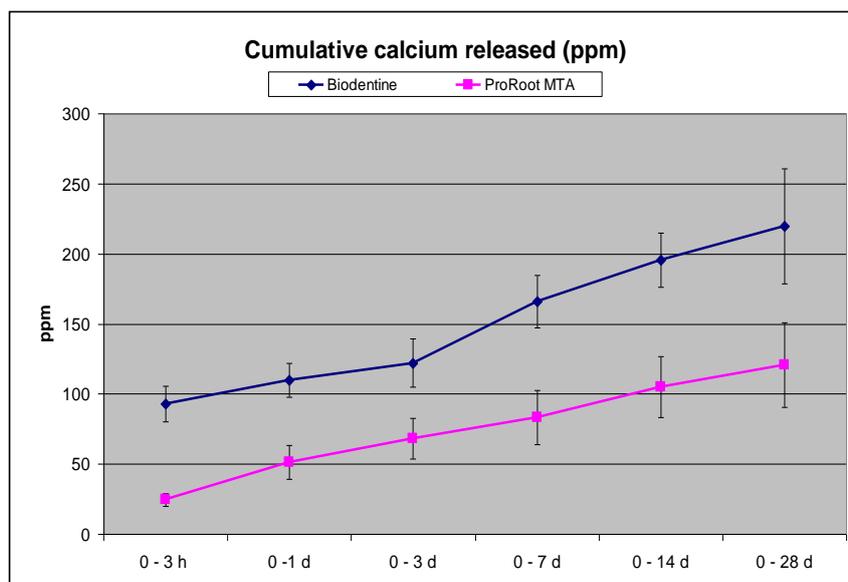
3.2. Physical Properties

3.2.1. Porosity

Significantly higher values of open porosity and apparent porosity were measured for ProRoot MTA (Table 1C) whilst the value of impervious (closed) pores was similar.

3.2.2. Setting Time, Solubility and Water Sorption

The setting time values differed widely between the materials (Table 1D). The water sorption values were similar but slightly higher for ProRoot MTA (14.55%) than for Biodentine (12.64%). The solubility values were also similar with no statistical difference, being 10.70% for ProRoot MTA and 11.93% for Biodentine.

3.3. Apatite-Forming Ability: ESEM-EDX Morphological and Chemical Surface Analysis

EDX analysis of the freshly prepared materials yielded the qualitative semi-quantitative elemental composition. Ca/P deposits differing in amount and size were detected by ESEM on the surface of the materials after immersion in HBSS.

Biodentine

Figure 2 reports the environmental scanning electron microscopy data and microanalysis of Biodentine. Freshly mixed Biodentine (Figure 2a1) showed a uniform surface containing interspersed granules, approximately 5 microns wide, and displayed Ca and Si from tricalcium silicate and Cl (6.91 wt%, from the aqueous calcium chloride). Zr (2.29 wt%), C (6.21 wt%), and N (3.34 wt%) reflexes (from components non indicated by the manufacturer, likely zirconium oxide, carbonates, and organic components) were also detected. No Al was present at any time (Figure 2a2,a3).

Figure 2. Environmental microscopy and microanalysis of Biodentine soaked in HBSS showing ESEM pictures (**a1**,**b1**,**c1**), EDX spectra of the whole area (**a2**,**b2**,**c2**), the elemental X-ray microanalysis (compositional weight % and atomic %) (**a3**,**b3**,**c3**) and the element mapping (**c5**–**c9**) performed on the 28-day-aged surface (**c4**). Freshly mixed Biodentine (**a1**) showed a uniform surface containing interspersed granules approx 5 microns wide, and mainly displayed Ca, Si, Zr, and Cl reflexes (**a2**). With soaking time in HBSS (**b1**,**c1**), a layer of globular CaP precipitates formed on the surface, the reflexes of N and Zr disappeared, the Si component decreased in intensity and Na, Mg, and P elements from HBSS became detectable (**b2**). The intensity of P reflexes increased with the soaking time (**b2**,**c2**) and consequently the Ca/P atomic ratio decreased (**b3**,**c3**). At 28 days, the Ca/P atomic ratio was approximately 2.5 (**c3**).

Element	Weight%	Atomic%
C K	6.21	11.83
N K	3.34	5.36
O K	35.23	50.38
Al K	0.01	0.01
Si K	6.90	5.62
S K	0.11	0.08
Cl K	6.91	4.46
Ca K	39.01	22.27
Zr L	2.29	0.57
Totals	100.00	
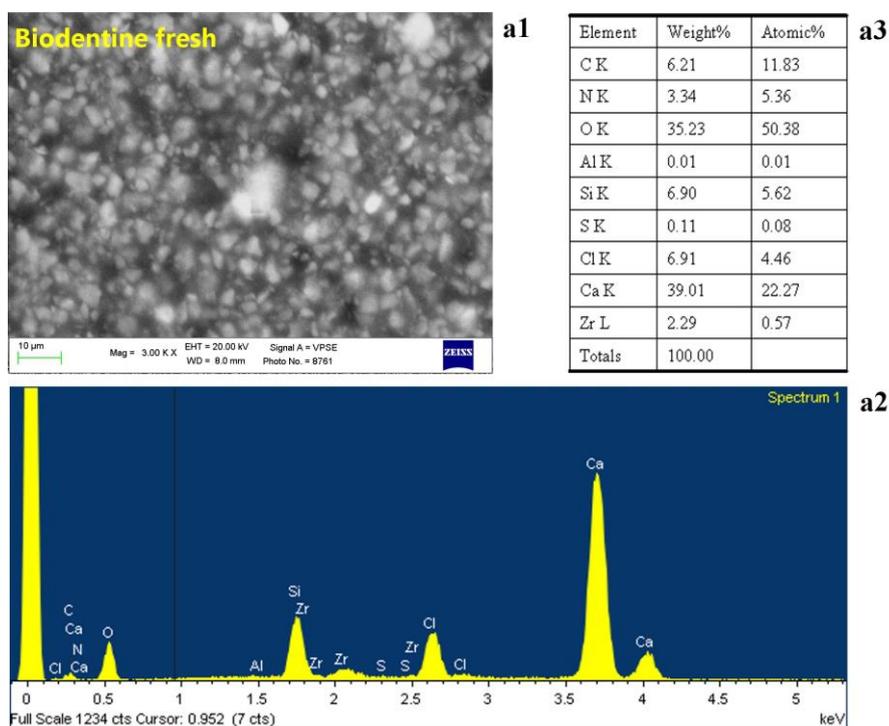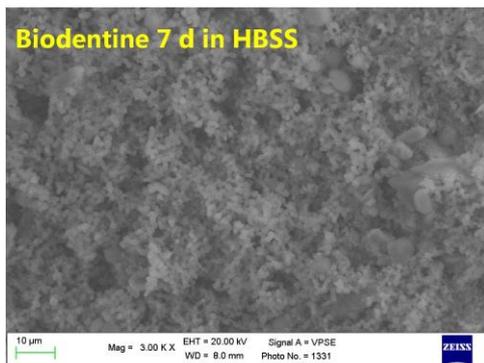
Element	Weight%	Atomic%
C K	6.43	10.67
O K	53.29	66.43
Na K	1.10	0.95
Mg K	2.84	2.33
Si K	5.53	3.93
P K	1.99	1.28
Cl K	0.98	0.55
Ca K	27.84	13.85
Totals	100.00	
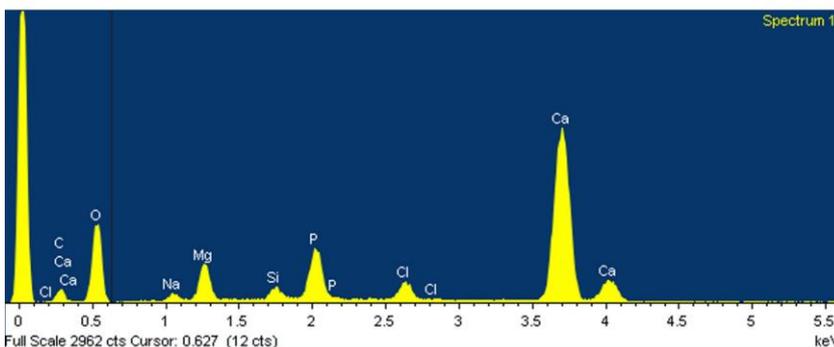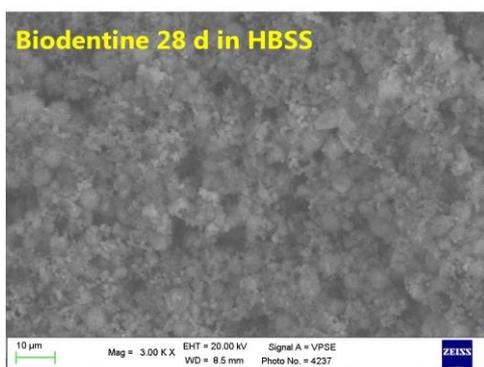
| Element	Weight%	Atomic%
C K	5.39	9.30
O K	50.97	66.03
Na K	0.66	0.60
Mg K	0.70	0.59
Si K	0.33	0.24
P K	9.91	6.63
Cl K	0.56	0.33
Ca K	31.49	16.29
Totals	100.00	
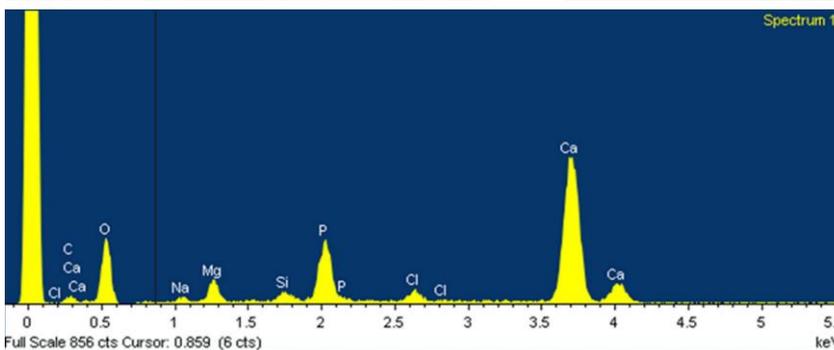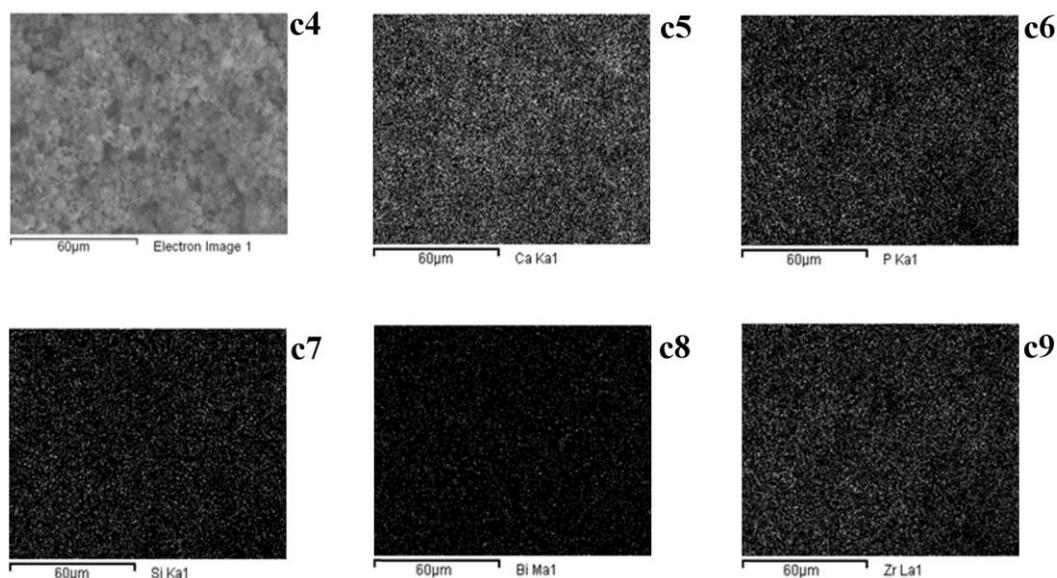
With soaking time in HBSS, the reflexes of N and Zr disappeared, the Si component decreased in intensity, and Na, Mg, and P elements from HBSS became detectable (Figure 2b2). The surface was covered by globular precipitates (Figure 2b1,c1) and P reflexes increased in intensity with time so that the Ca/P atomic ratio from 10.82 at seven days (Figure 2b3) decreased to 2.46 at 28 days (Figure 2c3).

Additional ESEM-EDX analyses on unhydrated powder (unreported data) revealed the following elements in weight %: C (4.34%), N (5.29%), O (42%), F (0.1%), Si (7.3%), Ca (39%), and Zr (2.2%) demonstrating the presence of organic additives and a radiopacifier in the anhydrous powder.

ProRoot MTA

Freshly mixed ProRoot MTA (Figure 3) displayed a finely granular surface revealing Ca and Si (from calcium silicates), Al (0.87% wt), and Bi (9.67 wt%, likely from calcium aluminate and calcium alumino-ferrite and bismuth oxide) and C (3.6 wt%). No S was detected at any time.

Figure 3. Morphochemical characterization of ProRoot MTA soaked in HBSS. ESEM image of freshly mixed ProRoot MTA (**a1**) displayed a finely granular surface and EDX analyses (micro-chemical spectrum and semi-quantitative compositional analysis) revealed Ca, Si, Al, and Bi (**a2,a3**). After soaking in HBSS (**b1**), the reflexes of the components Si and Bi decreased in intensity whilst P and C elements increased (**b2**). After 28 days the surface was coated by irregularly distributed globular CaP precipitates (**c1**), however, Bi was still detectable (**c2**). The semi-quantitative compositional analysis showed that the Ca/P atomic ratio decreased with time (**b3,c3**). At 28 days, the Ca/P atomic ratio was approximately 1.4 (**c3**).
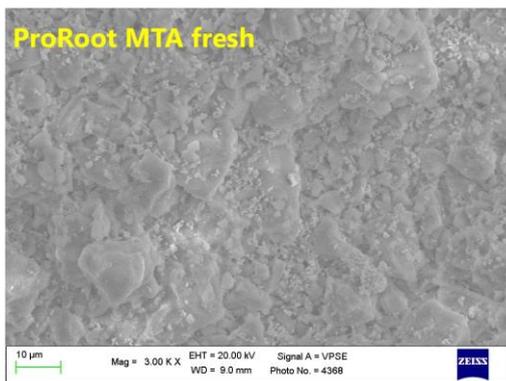
Element	Weight%	Atomic%
C K	3.60	7.19
O K	41.83	62.69
Al K	0.87	0.77
Si K	7.42	6.34
Ca K	36.60	21.90
Bi M	9.67	1.11
Totals	100.00	
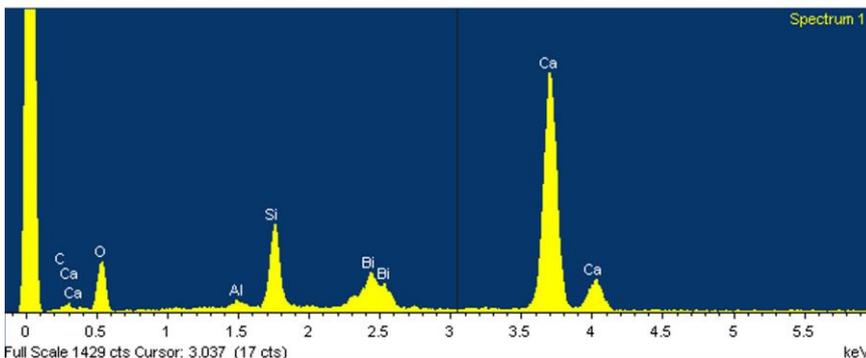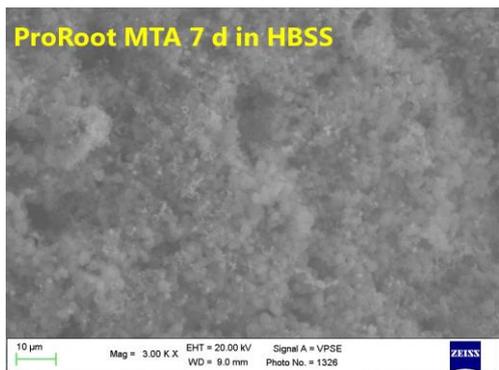
Element	Weight%	Atomic%
C K	8.55	13.45
O K	56.14	66.35
Na K	1.16	0.95
Mg K	7.60	5.91
Al K	0.93	0.65
Si K	2.11	1.42
P K	3.01	1.84
Cl K	1.67	0.89
Ca K	17.89	8.44
Bi M	0.94	0.08
Totals	100.00	

Element	Weight%	Atomic%
O K	39.59	59.88
Na K	2.75	2.89
Mg K	0.41	0.41
P K	19.05	14.88
Cl K	1.86	1.27
Ca K	33.72	20.36
Bi M	2.62	0.30
Totals	100.00	
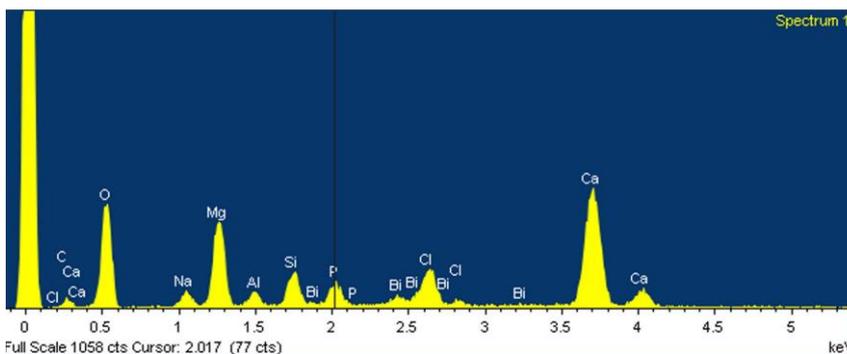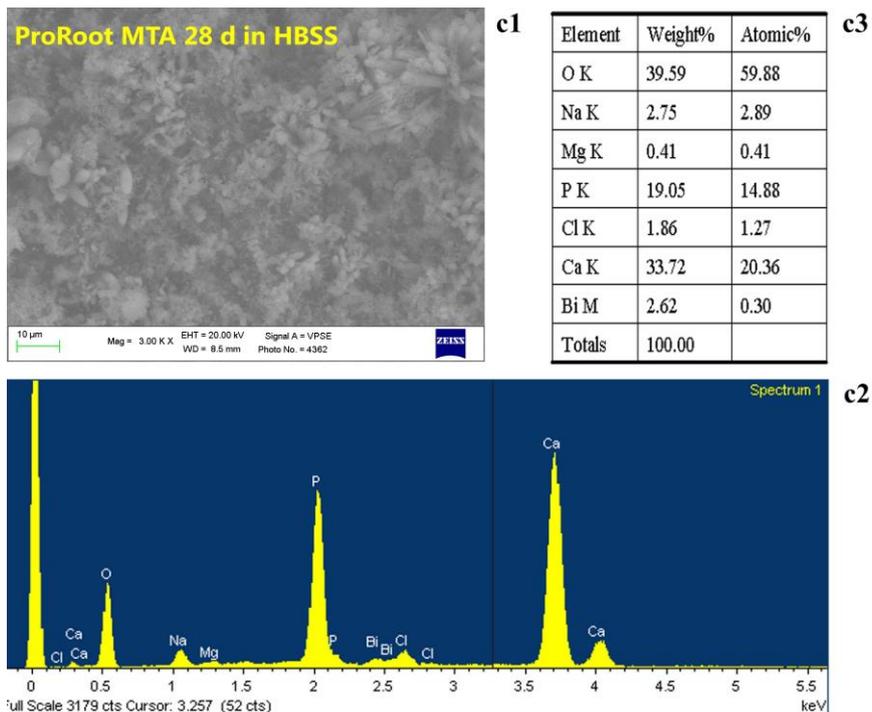
After seven days in HBSS, the P element and traces of Na and Cl (from the HBSS) were noted (Figure 3b2). A high Mg peak was detected. The reflexes of the components Si and Bi decreased in intensity whilst C increased (8.55 wt%), probably due to a carbonation phenomenon after soaking. No S was detected. The Ca/P atomic ratio was 5.32 (Figure 3b3).

Following 28 days in HBSS (Figure 3c1) the surface was coated by irregularly distributed globular CaP precipitates so Bi was still detectable (Figure 3c2). No S was found. The Ca/P atomic ratio was 1.37 (Figure 3c3).

4. Discussion

Calcium silicate materials are used in contact with periapical bone tissue or with vital pulp. For this reason they should possess specific bio-properties like biocompatibility, biointeractivity (ion-releasing, *i.e.*, release of biologically relevant ions), and bioactivity (apatite-forming ability) in order to promote the activity of mineralizing cells and the formation of new periapical bone or reparative dentine.

The present study tested a recent material introduced in the last two years (Biodentine, Septodont, Saint-Maur-des-Fossés, France) together with a popular calcium silicate-based formulation (ProRoot MTA, Dentsply Tulsa, Johnson City, TN, USA) comparing their chemical-physical properties (porosity, water sorption, solubility, and setting time) and the bio-properties of biointeractivity and bioactivity (release of calcium and hydroxide ions and apatite-forming ability). The study also evaluated the interlinking/correlation between the formation of calcium phosphate deposits on the materials surface and their specific chemical-physical properties. The null hypothesis of the absence of differences between Biodentine and ProRoot MTA properties has been disproved and should be rejected in favor of the alternative hypothesis testing that statistically significant differences exist.

Both materials showed good biointeractivity and the ability to form calcium phosphate deposits after soaking in simulated body fluid. Biodentine showed a markedly higher release of free calcium ions, higher alkalinizing capability, and the formation of smaller calcium phosphate deposits. Moreover, Biodentine demonstrated lower apparent porosity, volume of open porosity, and water sorption. Unexpectedly, despite such different setting times, Biodentine and ProRoot MTA showed similar solubility.

The ability to release calcium is a key factor for successful endodontic and pulp capping therapies because of the action of calcium on mineralizing cells (osteoblasts, cementoblasts, pulp cells, and odontoblasts) differentiation and hard tissue mineralization [9,14,15,33–39].

Ca ions are necessary for the differentiation and mineralization of pulp cells [34] and Ca ions specifically modulate osteopontin and bone morphogenetic protein-2 levels during pulp calcification [33]. In addition, the eluted Ca ions increase the proliferation of human dental pulp cells in a dose-dependent manner [40,41] and Ca release enhances the activity of pyrophosphatase, which helps to maintain dentine mineralization and the formation of a dentine bridge [42].

Moreover hydroxide ions stimulate the release of alkaline phosphatase and BMP-2, which participate in the mineralization process [13,14]. Several studies demonstrated calcium silicate-based materials share their properties to induce the proliferation and genes activation of periodontal fibroblasts [43], dental pulp cells [13,44–46], cementoblasts [47], osteoblasts [14], mesenchymal stem cells [10,11], and to stimulate the reparative hard tissues [6,7].

Calcium release and pH were measured in distilled water rather than simulated body fluid in order to standardize the test conditions and hence allow a comparison of the data with other future studies.

The ability of Biodentine to release biologically relevant ions potentially acting as epigenetic chemical signals involved in the activation of mineralizing bone/dentine-forming cells is in agreement with recent studies demonstrating the inductive effects of Biodentine on gene expression by pulp cells and odontoblasts [44–46].

Ion release depends on several factors such as the nature of the network structure and the mineral particles responsible for water sorption and solubility as well the permeability of the material to water diffusion (*i.e.*, porosity).

Biodentine showed a markedly higher release of free calcium ions compared with ProRoot MTA, higher alkalinizing capability and the formation of well-defined smaller (<1 micron) spherulites of calcium phosphate forming a more dense and compact coating on its surface, whilst ProRoot MTA showed the formation of rounded agglomerates 1−5 micron in diameter.

ESEM analyses of fresh Biodentine showed a surface strongly different from that of ProRoot MTA, with particles appearing suspended in a matrix. EDX displayed Ca and Si due to the tricalcium silicate component, Cl from calcium chloride likely inserted to shorten the setting time [48] and C likely from calcium carbonate. Both C and N (3.34% wt) were probably from (polyamino) carboxylates that could be present as a dispersant and water reducer (the mixing fluid was present only as 20.45% of the final paste). The detection of Zr (2.29% wt) was inconsistent with the stated composition indicating undisclosed ingredients, likely zirconium oxide added as a radiopacifier and possibly tricalcium aluminates. S (correlated with the presence of sulphates) and Al elements were not detected. Additional microanalyses performed on unmixed calcium silicate powder also disclosed C, N, and Zr indicating that the supposed organic and radiopacifying components are ingredients of Biodentine powder. S was never detected in Biodentine indicating the probable absence of a calcium sulphate component typical of Portland cement.

The high Ca release of Biodentine can be correlated with the presence of a calcium silicate component and calcium chloride and low solubility (11.93%) likely linked to a superplasticizer like polycarboxylate commonly used to reduce the amount of water required (L/P 0.257) to disperse the particles and to enhance the fluidity, making the cement self-consolidating. In addition, the fast hydration reaction of tricalcium silicate can be correlated with the low solubility and high calcium release at early endpoints (a few h).

The large amount of calcium released favors the formation of CaP deposits and a coating composed of small CaP was evident on the Biodentine surface after soaking. However, the high Ca/P atomic ratio (approximate 2.46) obtained at 28 days, together with the detection of a Si component, may be caused by the thin or uneven CaP coating, and the presence of Ca-rich calcium phosphates and calcium carbonate (and/or carbonated phases). Due to its favorable biological properties (biocompatibility, osteoconductivity, bioresorption, and biomineralization) [49,50], calcium carbonate could have beneficial effects on mineralizing cell activation. Calcium carbonate is one of the most abundant and important biominerals in nature (it is deposited by osteoblasts together with calcium phosphates during the mineralization process) and both natural and synthetic forms are now used as biomaterials for tissue engineering.

The mechanism of apatite formation on calcium-silicate MTA cements in phosphate-containing solutions was summarized by Gandolfi *et al.* in 11 steps [21]. The growth of a layer of apatite is an ideal environment for stem cell and osteoblasts differentiation and colonization, and to support new bone formation [31]. The apatite-forming ability together with the epigenetic signals correlated to ion release may well explain the excellent clinical outcomes of MTA cements [6].

In the present study both calcium silicate materials showed high biointeractivity (ion release) and fast formation of apatite with remarkable open pore volume and apparent porosity. The high open pore

volume forms an internal network of water-filled pores providing a large surface area involved in the leaching process. The Ca/P atomic ratio of the coating (ranging approximately 1.4–2.5) approached that of bone-like carbonate apatite.

Both calcium silicate materials leached large amounts of Ca and OH ions: the hydration reaction of the calcium silicate particles triggers the dissolution of their surface with the formation of a calcium silicate hydrate gel and $Ca(OH)_2$, together with the release of Ca and OH ions.

Although Biodentine and ProRoot MTA have a similar calcium silicate composition, their ion-releasing capacity, setting times (12 min for Biodentine and 170 min for ProRoot MTA) and porosity differed widely, likely due to chemical and particle size differences in the calcium silicate mineral powder. Unexpectedly, their solubility and water sorption were similar. The porosity and water uptake tests in the present study were performed using Archimedes' Principle, as proposed by ASTM C373-88 and following ISO 3107, and values concerning the volume of open and closed porosities were obtained.

The open porosities and apparent porosity can be correlated with a pronounced ion release: the large open pore volume and water sorption provided a broad wet biointeractive surface for the release of the calcium and hydroxyl ions involved in the formation of a calcium phosphate coating.

The solubility test in the present study was performed following ISO 4049 calculating the decline in mass (weight loss) after storage in deionized water [18,51,52], and the results expressed as a percentage of the specimen's original weight. The test measures the elution of water-soluble material and disintegrated portions. The "high solubility" of calcium silicate-based materials can mainly be explained by the formation, during the hydration and setting reactions, of soluble calcium salts and calcium hydroxide, which are rapidly washed out by water.

In the present study Biodentine showed a solubility value (11.93%) higher than that stated by the manufacturer's brochure (6.8%). This lower solubility of Biodentine compared with ProRoot MTA can be correlated with the restricted amount of the mixing fluid likely containing water-reducer agents (such as polycarboxylic ether), as suggested by EDX data and by the patent, an idea taken from building materials (concrete).

Evaluation of the results must take into account that both calcium silicate-based materials were prepared, following the manufacturer's directions, by mixing the mineral powder with a liquid (water or water-based liquid) using different liquid-to-powder weight proportions for each material, namely 0.26 for Biodentine and 0.31 for ProRoot MTA. This means that a prominent variable percentage of the initial material mass was constituted by mixing liquid, namely water (ProRoot MTA 23.66 wt%) or water-based (Biodentine 20.45 wt%) susceptible to evaporation in the drying procedure needed for the solubility test to obtain the final dry mass.

Moreover, the hydration of calcium silicate cements proceeds by converting liquid (free) water (*i.e.*, the mixing water) into structural and constrained water. Tricalcium silicate (the Biodentine component) is very reactive and hydrates rapidly (heat peak of the hydration reaction at approx. 10 h) whereas dicalcium silicate (the main ProRoot MTA component together with tricalcium silicate) hydrates slowly [53,54]. The solubility tests were performed after a period equal to 70% of the setting time of each material, *i.e.*, 250 min for ProRoot MTA and nine min for Biodentine. At these times, the hydration reaction of ProRoot MTA is at an early stage and only a small part of the liquid free water can be converted into structural and constrained water, and most of the water can still be removed by

the test drying procedure at 37 °C providing a high weight loss that does not truly correspond to the true solubility of the material. Conversely, due to its fast hydration rate, tricalcium silicate solidifies rapidly providing a good early strength to the paste responsible for the low solubility obtained for Biodentine.

In addition, the solubility data of the water-containing calcium silicate-based cements can also be overestimated referring to the clinical condition where these biomaterials benefit greatly from the presence of body fluids, and the formation of calcium phosphate deposits on their surface and inside the porosities [18,55] can improve their mass. Moreover, in the presence of biological fluids in a clinical situation, the leaching of water-soluble components and the correlated weight loss is lower than in deionized water.

The limitation of this *in vitro* study is the use of distilled water as testing medium, in agreement with the international regulations, for the measurements of the chemical-physical properties in order to standardize the test conditions and hence allow a comparison of the data with other future studies. The use of simulated body fluid should be considered more appropriate as biological fluids are in contact with such materials in a clinical condition, but it could provide misleading results. We previously reported [18] that solubility test performed in DMEM used as SBF caused the increase of the weight due to the fast precipitation of calcium phosphate deposits induced by the calcium ions released from the materials. Thus, in SBF is not possible to detect the real calcium release as many ions precipitates promoting the calcium leaching process from the material (pilot experiments of Biodentine immersed in HBSS showed the release of 157, 142, 150 and 14 ppm of calcium at 3h, 1, 7 and 14 days respectively). Similar considerations for the pH measurements, as is not possible to measure the real alkalinizing activity of a material in SBF in relationship to the presence of buffering activity.

5. Conclusions

Biodentine is a fast-setting tricalcium silicate-based material having extended alkalinizing properties principally able to release ions involved in mineralization processes. The material's large porosity and water sorption are correlated with a pronounced ion release allowing the formation of calcium phosphate deposits. Biodentine therefore seems an alternative to MTA, but further research is required.

Acknowledgments

Conflicts of Interest

The authors declare no conflict of interests.

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