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Bioactivity potential of Portland cement in regenerative endodontic procedures: From clinic to lab

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ABSTRACT

Objective. The aim of this study was to evaluate the bioactivity potential of an hydraulic calcium-silicate cement, Pure Portland Cement Med-PZ (Medcem, Weinfelden, Switzerland: 'MPC'), applied in a tooth extracted because of failed regenerative endodontic procedures (REP) and by means of *ex vivo* (EV) specimens.

Methods. Ten EV cylindrical dentin cavities were prepared and filled with MPC and stored for 1 month in distilled water (DW), Hank's balanced salt solution (HBSS), Dulbecco's phosphate-buffered saline (DPBS), simulated body fluid (SBF), *versus* no media (NM) serving as control. Six additional EV specimens were filled with MPC and exposed for 2 weeks to leucocyte-and-platelet-rich fibrin (LPRF)-clot (C), LPRF-membrane (M) and LPRF-exudate (E). MPC in the EV specimens and in the coronal part of the REP tooth was analyzed by means of micro-Raman spectroscopy (MR), scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS).

Results. SEM showed rough crystallite surfaces for the EV samples and a porous surface for the REP tooth. EDS of the EV samples revealed prominent peaks for Ca, Si and O. Storage in HBSS, DPBS, SBF, exposure to LPRF and the REP tooth showed considerable amounts of P as well. MR exhibited vibrations of phosphate (DPBS, SBF), carbonated hydroxyapatite (DPBS, SBF), calcium carbonate (DW, HBSS, NM, REP-tooth, LPRF-E), oxidized (ferric) proteins (LPRF-E/C/M) and the amide III band (all samples). Hence, only storage of MPC in DPBS and SBF for 1 month revealed bioactivity.

Significance. The environmental conditions, namely the laboratory and clinical settings, affect the bioactivity potential of MPC.

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1. Introduction

Infected immature permanent teeth with pulp necrosis are more recently treated with regenerative endodontic procedures (REP) [1,2]. This novel endodontic treatment modality is a biologically based treatment that aims to restore the function and further development of the damaged pulp-dentincomplex [3]. After disinfection of the root canal during the first REP session, bleeding is provoked periapically at the second REP session to induce the formation of a blood clot inside the root canal and to attract stem cells that might aid further root development. Additionally, 2 other pillars of tissue engineering are introduced during REP, namely the placement of a scaffold (in which the stem cells might nestle) and the administration of growth factors (aiding the healing process) [2]. Many scaffolds have been proposed and are being developed [4]. The ideal scaffold for biomedical application should be biocompatible, biodegradable and mimic the extracellular matrix (ECM). On one hand, synthetically-engineered polymeric and ceramic scaffolds seem to give promising results in terms of bone regeneration. On the other hand, the degradation of these synthetic scaffolds might be a concern and they lack the physiological information that is available in the ECM [4]. However, post REP, rather pulp and dentin are appreciated inside the root canal than bone. Natural scaffolds such as an autologous platelet concentrate have been applied in the medical and dental field for decades [5,6], among which leucocyte-and-platelet-rich fibrin, commonly referred to as 'LPRF', seemed to reveal one of the most favorable outcomes in terms of regenerative wound healing [7]. An autologous scaffold loaded with growth factors is most ethical and cost-effective. In the field of REP, LPRF has been implemented with promising results in terms of periapical bone healing, further root development [8] and regaining pulp sensibility [9]. The LPRF-clot has a specific anatomy (Fig. 1) with underneath the red thrombus a buffy coat, in which most of the platelets, leukocytes and growth factors are entrapped [7]. This LPRF-clot is also very plastic and can be adapted to the operation site (*e.g.* root canal). More specifically, it can be transformed into a membrane by squeezing the exudate out by means of a sterile glass plate (Fig. 1) [10].

During the second REP session, a hydraulic tricalcium silicate-based cement (TCS) is placed upon the blood clot and/or scaffold to seal the root canal [1,2]. So-called mineral trioxide aggregate (MTA) has been one of the most investigated TCSs [11]. However, MTA may discolor the tooth due to bismuth oxide [12], by which it is not favorable to be applied coronally [13]. Pure Portland Cement Med-PZ (Medcem, Weinfelden, Switzerland: 'MPC') has similar chemo-mechanical properties as MTA and does not contain bismuth oxide [14]. A particular property of TCS is its bioactivity potential [15,16]. As the TCS in REP are in close contact with blood, synthetic/natural scaffolds and even dental pulp in some cases, it should reveal a biological response. After all, a bioactive material has the ability to induce a specific biologic activity [17]. In the field of tissue engineering, this definition has undergone a slight modification, namely that a bioactive material will form carbonated apatite after being immersed in a serum-like solution (such as simulated body fluid (SBF)) [18]. During and after setting in REP, TCS remains in contact with blood and tissue fluids. Ageing of TCS for endodontic applications in SBF have already been tested *in vitro* [19] and even if an ISO standard (ISO 23317(2012)) has been prescribed to test the apatite forming ability of Portland cement *in vitro* [18], the clinical situation is not comparable to the lab setting. The TCS investigated in human dentin slices of extracted teeth (where cement is put into the dentin slices in the lab) undergoes other manipulations and environmental conditions than when it has been put into a patient's root canal during treatment [20]. Furthermore, the type of (endodontic) treatment modality is another factor. TCS put as a root-end filling during apexification or apex-resection [21] is much nearer to periapical medullary bone [22] than when the TCS is put directly below the cemento-enamel junction in REP, which directly influences the apatite-forming ability of the TCS. Thus, specific environmental conditions may modify the material setting [23–25]. Very little information is available on the environment TCS is placed in during REP and almost nothing is known on the material chemistry of failed clinical cases [21]. Hence, the purpose of this study was to investigate the effect of environmental conditions on MPC bioactivity by analyzing an extracted tooth after REP failure posttrauma and comparing it with *ex vivo* (EV) specimens. To our knowledge no previous study has reported the chemical and micro-structural analysis of Portland cement applied in a patient's REP-treated tooth.

2. Methods

This study was designed as an *in vitro* (IV) study and was conducted according to the Good Clinical Practice standards (International Council on Harmonization, 1996), which rely on the ethical principles of the Declaration of Helsinki (World Medical Association, 1964). Ethical approval was obtained by the Medical Ethics Committee of UZ/KU Leuven under the file number S60773. One patient's sample and 16 EV samples placed in different media were investigated by means of micro-Raman spectroscopy (MR), scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS). MR is a spectroscopic technique commonly used in chemistry to provide the structural fingerprint of a material in a non-destructive manner [26].

2.1. *Patient's sample*

2.1.1. *Clinical part*

The right and left central maxillary permanent incisors of an eight-year-old girl suffered an uncomplicated crown fracture. Both teeth reacted positively to carbon dioxide snow (CDS) and were restored with a calcium-hydroxide lining (Life, Kerr, Scafati, Italy), glass-ionomer cement (Fuji 2 LC, GC, Tokyo, Japan; 'GIC') and composite (Filtek Supreme XTE, 3M Oral Care, Seefeld, Germany). Nevertheless, 8 months later, the left central maxillary permanent incisor reacted negatively to CDS and presented a vestibular abscess. The tooth received REP following the procedure detailed by the European Society of Endodontology [1], with the exception that no CollaPlug (Zimmer Biomet, Berlin, Germany) was used underneath the MPC and a zinc phosphate cement (De Trey Zinc, Densply Sirona,

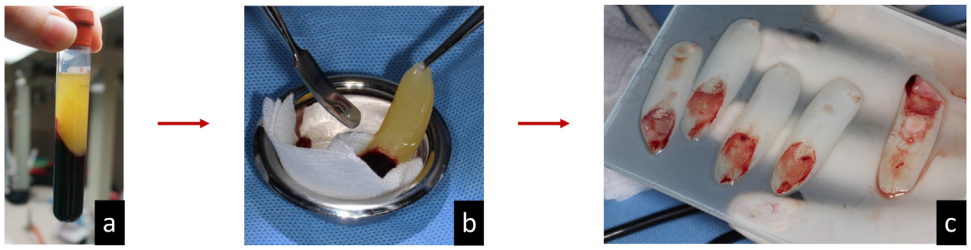

Fig. 1 – Leucocyte-and-platelet-rich fibrin (LPRF): (a) LPRF in a sterile tube post centrifugation; (b) LPRF-clot; (c) LPRF-membrane.

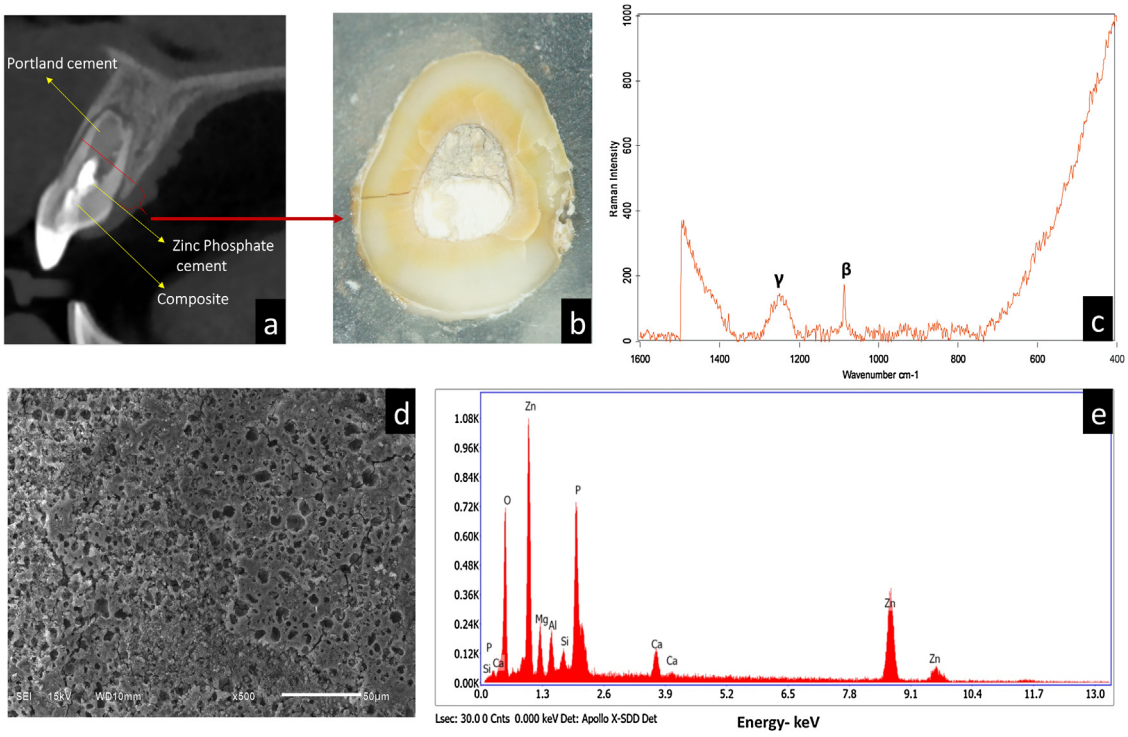

Fig. 2 – Patient's case: (a) Conebeam CT 22 months post regenerative endodontic procedures of the left central maxillary permanent incisor; (b) Cylindrical dentin slice with a 2-mm thickness, containing a Pure Portland Cement Med-PZ (Medcem, Weinfelden, Switzerland; MPC) root-canal filling in the center; (c) micro-Raman analysis of the filling material in (b), revealing a β-calcium carbonate peak at 1082 cm^{-1} and a γ-peak for the amide III band (N—H) at 1242 cm^{-1}; (d) SEM micrograph of the filling material in (b); (e) EDS of the filling material in (b).

Konstanz, Germany) was placed instead of GIC underneath the composite restoration. At the recall 20 months post REP, the periapical lesion was healed, but not enough root-canal wall thickening was obtained (Fig. 2b). After multi-disciplinary consultation at 22 months post REP, it was decided to remove this tooth rather than extracting a sound premolar to create space for orthodontic reasons. Written informed consent was obtained by the patient and the parents to use the tooth for further research.

2.1.2. *Tissue processing*

Directly after extraction, the REP tooth was sectioned using a diamond saw (Isomet 1000, Lake Bluff, IL, USA) (Fig. 2b). The coronal part was kept in a carbon dioxide-free environment (= desiccator with soda lime and silica gel) for 24 h. The apical part of the tooth was fixed using 6% formaldehyde and processed for immunohistology (data not in the scope of this study). Afterwards, the coronal part of the REP tooth was imbedded in resin (Unifast III, GC) and segmented with a microtome (Micracut 151, Metkon, Bursa, Turkey) into a cylindrical dentin slice with a 2-mm thickness, containing MPC in the central root-canal space (Fig. 2b). Subsequently, the specimen was processed for SEM by fixation in 2.5% glutaraldehyde for 24 h, dehydration in ascending concentrations of ethanol (25, 50, 75, and 95% for 30 min each, and 100% for 1 h), and finally drying by immersion in hexamethyldisilazane for 10 min. Prior to further SEM processing, MR was performed using a Senterra device (Bruker, Optik, Ettlingen, Germany). MPC was scanned on 9 randomly selected spots using a near-infrared (785 nm) laser with 50 mV power, a ×50 microscope objective and 50 × 1000 aperture, and an integration time of 10 s with 3 co-additions. The CCD detector possessed a 1024 × 256 pixel resolution and was cooled down thermo-

electrically to −65 °C. MR peaks characteristic for MPC were recorded. Post-processing of the spectra was performed using the Opus Spectroscopy Software version 7.0 (Bruker, Ettlingen, Germany). Subsequently, the specimen was mounted by means of a double-sided carbon tape on an aluminum stub, carbon coated and gold-sputtered by means of a JEOL JFC-1300 auto-fine coater (Jeol, Tokyo, Japan). SEMs were taken using a FEI XL30 field-emission-gun (Feg)-SEM (FEI, Eindhoven, The Netherlands) employed in the secondary electron mode at different magnifications, a voltage of 15 kV and a 10-mm working distance. At a magnification of 500× and a voltage of 20 kV, the EDS detector (EDAX, Tilburg, The Netherlands) of the FEI XL30 Feg-SEM (FEI) was used to chemically map the surface composition of the selected area.

2.2. *Ex vivo samples in different media*

2.2.1. *Storage media*

Eight human central incisors (gathered as approved by the Medical Ethics Committee of UZ/KU Leuven under the file number S60773) were stored in 0.5% chloramine solution at 4 °C and were used within 3 months after extraction. The teeth were embedded in resin (Unifast III, GC) and segmented with a microtome (Micracut 151, Metkon) into 16 cylindrical dentin slices (2 per tooth) with a 2-mm thickness. At the root-canal region of the dentin slices, a cavity with a diameter of 2 mm was prepared using a diamond bur (Komet, Lemgo, Germany). MPC (Medcem; lot-number: MWZ170215) was prepared strictly following the manufacturer's instructions and placed into the central cavity of the EV specimens by means of an amalgam gun. The resin border of each specimen was marked with a bur for upward orientation. Prior to setting, the EV samples were immersed in 25 mL of the following media (2 samples per media) with the resin mark pointing upwards: distilled water (DW), Hank's balanced salt solution (HBSS), Dulbecco's phosphate-buffered saline (DPBS), simulated body fluid (SBF), *versus* no media (NM) serving as control. The EV samples were stored in an incubator at 37 °C for 1 month with the media changed every 2 weeks. SBF was made by the main investigator (MN) and consisted of 136.8 mM NaCl, 4.2 mM $NaHCO_3$, 3.0 mM KCl, 1.0 mM $K_2HPO_4{\cdot}3H_20$, 1.5 mM $MgCl_2{\cdot}6H_2O$, 40 mM HCl, 2.5 mM$CaCl_2$, 0.5 mM Na_2SO_4, 50 mM $(CH_2OH)_3CNH_2$; its pH was adjusted to 7.4 using a pH 3110 Set VWR pH meter (WTW, Weilheim, Germany).

2.2.2. *LPRF*

Furthermore, a venipuncture on the median cubital vein of MN was performed by a nurse (UZ Leuven, Leuven, Belgium) and blood was drawn into 2 sterile, 10 mL plastic tubes without anticoagulant. The tubes were immediately centrifuged at 702 RCF in an IntraSpin centrifuge (Intra-Lock International, Boca Raton, FL, USA) to obtain LPRF-clots (Fig. 1). One clot was pressed for 5 min under a sterile glass plate, in order to obtain LPRF-membranes and -exudate (Fig. 1). After mixing, but prior to MPC setting, 4 separate EV specimens were placed in contact with LPRF-clots (n = 2) and LPRF-membranes (n = 2), while 2 additional EV specimens were immersed in LPRF-exudate. All LPRF-specimens were placed for 2 weeks in the incubator at 37 °C, as LPRF loses its functionality after 7–14 days [7]. Prior to analysis, the specimens were retrieved and placed in a carbon dioxide-free environment (= desiccator with soda lime and silica gel) for 24 h. Sample preparation prior to analysis and the analyses themselves were performed as described for the patient's sample. Per medium 1 EV sample was used for MR and another was further processed for Feg-SEM and EDS.

3. Results

3.1. *Patient's sample*

SEM revealed a rough porous surface (Fig. 2d). EDS showed high concentrations of zinc, phosphorus and oxygen, and lower amounts of magnesium, aluminum, silicon and calcium (Fig. 2e). The relative weight percentage of the elements is presented in Table 1. MR exhibited vibrations of the amide III band (N—H) at 1242 cm^{-1} [27] and calcium carbonate at 1082 cm^{-1} [26,28] (Fig. 2c).

3.2. *Ex vivo samples*

3.2.1. *Five different media*

SEM overall presented rough surfaces (DPBS, SBF; Fig. 3h,k) and some samples presented crystals (DW, HBSS, NM; Fig. 3b,e,n). This difference in microstructure depending on the storage media is related to the discrepancies found in the chemical surface analysis. On the one hand, EDS for DW, DPBS and NM (Fig. 3c,i,o) showed high calcium and silicon peaks (>7,6 K) and intermediate-to-small peaks representing oxygen, sodium, aluminum and magnesium (<475 K). On the other hand, HBSS and SBF presented similar chemical compositions, being more specifically rich in phosphorus, oxygen and calcium (Fig. 3f,l). Phosphorus was also present when stored in DPBS (Fig. 3i). Hence, the phosphate peak at 960 cm^{-1} was also detected by MR when the EV samples had been stored in DPBS and SBF (Fig. 3p). MPC in all groups presented vibrations for the amide III band (N—H) at 1242 cm^{-1} (γ-peak in Fig. 3p) [27]. Furthermore, DPBS and SBF storage resulted in a vibration of the carbonate group (β-type of carbonate) in hydroxyapatite at 1070 cm^{-} [26,28,29]. HBSS, DPBS and NM revealed a calcium-carbonate peak with high Raman intensity at 1082 cm^{-1} [26,28].

3.2.2. *LPRF*

MPC exposed to LPRF-clot was greyish discolored (Fig. 4

a) versus yellowish discolored when having been exposed to LPRF-membrane (Fig. 4e) and -exudate (Fig. 4i). SEM of the sample exposed to LPRF-clot presented a rough and granular surface with deposits (Fig. 4b,c), while the sample exposed to LPRF-membrane showed a rough structure with round crystals (Fig. 4f,g). These granular desposits and crystals were rich in calcium, but they also revealed a phosphorus peak (Fig. 4d,h,l) implicating the presence of phosphate.

MR of MPC exposed to LPRF-clot and -membrane presented similar vibrations for the amide III band (N—H) at 1242 cm^{-1} [27] and a peak at 1370-1378 cm^{-1} representing oxidized (ferric) proteins [30] (Fig. 4m). Nevertheless, EDS of MPC exposed to LPRF-clot presented 2.40 wt% of iron (Table 1). The other EDS peaks for LPRF-clot and -membrane were similar, namely high carbon, oxygen, calcium and silicon peaks and moder-

Table 1 – Elemental composition (wt%) of Pure Portland Cement (Med-PZ, Medcem, Weinfelden, Switzerland) stored following the different experimental conditions.

Chemical element	Patient's sample	Distilled water	HBSS	DPBS	SBF	No media	LPRF-C	LPRF-M	LPRF-E
OK	28.95	43.94	46.52	46.49	41.68	37.94	38.99	39.71	35.67
ZnL	40.68	–	–	–	–	–	–	–	–
MgK	6.63	1.68	–	2.67	–	0.21	0.89	0.80	1.03
AlK	4.34	5.74	–	5.17	–	0.25	3.33	2.82	1.21
SiK	2.23	17.18	–	24.51	0.59	10.68	20.03	11.92	2.78
PK	14.18	–	4.05	2.99	14.50	–	4.56	2.94	2.27
CaK	2.97	11.88	49.43	15.47	42.17	28.63	13.05	21.18	28.21
CK	–	9.69	–	–	–	21.99	15.20	18.50	27.97
NaK	–	2.46	–	2.70	1.07	0.30	1.55	2.13	0.86
FeK	–	1.49	–	–	–	–	2.40	–	–

C = clot; DPBS = Dulbecco's phosphate-buffered saline; E = exudate; HBSS = Hank's balanced salt solution; LPRF = leukocyte and platelet rich fibrin; M = membrane; SBF = simulated body fluid.

ate aluminum, magnesium, sodium and phosphorus peaks (Fig. 4d,h).

SEM of MPC exposed to LPRF-exudate showed a rough surface with clusters of acicular (needle-like) and round crystals (Fig. 4j,k). No iron was detected by EDS, but the spectra revealed high carbon, oxygen and calcium peaks and moderate aluminum, magnesium, phosphorus, silicon and sodium peaks (Fig. 4l, Table 1). MR of MPC exposed to LPRF-exudate was similar to that exposed to LPRF-clot and -membrane except for the calcium carbonate peak at 1082 cm^{-1} [26,28]. This mixed presence of phosphate and calcium carbonate explains the mixed crystal structure imaged by SEM.

4. Discussion

Portland cement is produced by burning limestone and clay in a kiln, which results in a cement clinker. This clinker is ground to a fine powder with gypsum, resulting in Portland cement. MPC consists mainly of tricalcium silicate and dicalcium silicate, and is classified as a hydraulic cement as it sets in the presence of moisture [15,16]. Hydrated MPC's end products are mainly calcium-silicate hydrate, calcium hydroxide and ettringite. About one third of the volume of hydrated MPC consists of calcium hydroxide that can be released [31]. Calcium hydroxide leads to an alkaline pH and induces apatite formation when MPC is immersed in physiological-like phosphate solutions [32–35]. A bioactive material has the potential to elicit a response from a living tissue or cells [17]. Hydraulic TCSs are regarded as bioactive materials because of their ability to induce hydroxyapatite formation [15]. Hydroxyapatite formation has to date only been observed in *in vitro* samples [34,35]. Research performed on human specimens retrieved from the root end [21] revealed only crystalline calcium carbonate in the presence of blood. In previous studies, only X-ray diffraction and EDS were used to analyze the retrieved materials. MR has already been applied to study *in vitro* the ageing of TCSs in SBF [19] and remineralization of demineralized human dentin slices when put in close contact to calcium silicate/hydroxide cements in physiological-like solutions [36]. However, until now, no MR-study has investigated TCS put for several months in the coronal part of the root of a patient's tooth. This is the first paper reporting the use of MR, SEM and EDS to analyze MPC retrieved from a patient's tooth due to failed REP posttrauma.

Physiological-like phosphate solutions such as SBF, HBSS and DSPS were applied in the current study as ageing media for MPC in order to mimic the clinical situation (patient's sample and LPRF-specimens) [32–35]. SBF is an acellular storage medium with ion concentrations equal to those of human blood plasma [18] and demands preparation, as performed in the current study by the principal investigator (MN). However, there are also commercially available media that mimic the ion composition of human blood plasma, such as HBSS and DPBS.

EDS was applied to chemically analyze the elemental composition of the specimens. Nevertheless, quantitative data obtained by EDS should be interpreted with caution as they are less accurate in comparison with wavelength spectroscopy [37]. The high EDS-peaks for calcium, oxygen and silicon for the MPC samples exposed to DW, HBSS, DPBS, NM and LPRF were characteristic for MPC, as MPC releases calcium-silicate hydrate and calcium hydroxide during setting [31]. Moreover, calcium was present in all specimens. Furthermore, MPC exposed to HBSS, DPBS, SBF and LPRF showed a considerable amount of crystals rich in phosphorus (Table 1), indicating the occurrence of phosphates. Phosphorus was also detected by MR; more specifically DPBS and SBF presented a phosphate peak at 960 cm^{-1} and a vibration of the carbonate group (β-type of carbonate) in hydroxyapatite at 1070 cm^{-1}, which is in accordance with previous studies [32,33]. Such MR vibration was missing for MPC exposed to DW and NM, due to the lack of phosphorus. Even if phosphorus was present in MPC exposed to LPRF and the patient's sample, rather calcium carbonate than phosphate and/or apatite was detected. This must be attributed to contamination with blood [21,33,38]. Regarding HBSS, as mentioned in previous studies, a storage time longer than one month is necessary to detect a higher bioactivity of TCS immersed in HBSS, due to the lower phosphate concentration of HBSS in comparison with DPBS [19,33]. Gandolfi et al. observed apatite formation only after 180-day storage of TCS in HBSS [33]. In the current study, MPC exposed to HBSS presented a calcium-carbonate peak due to the reaction of calcium hydroxide with environmental carbon dioxide

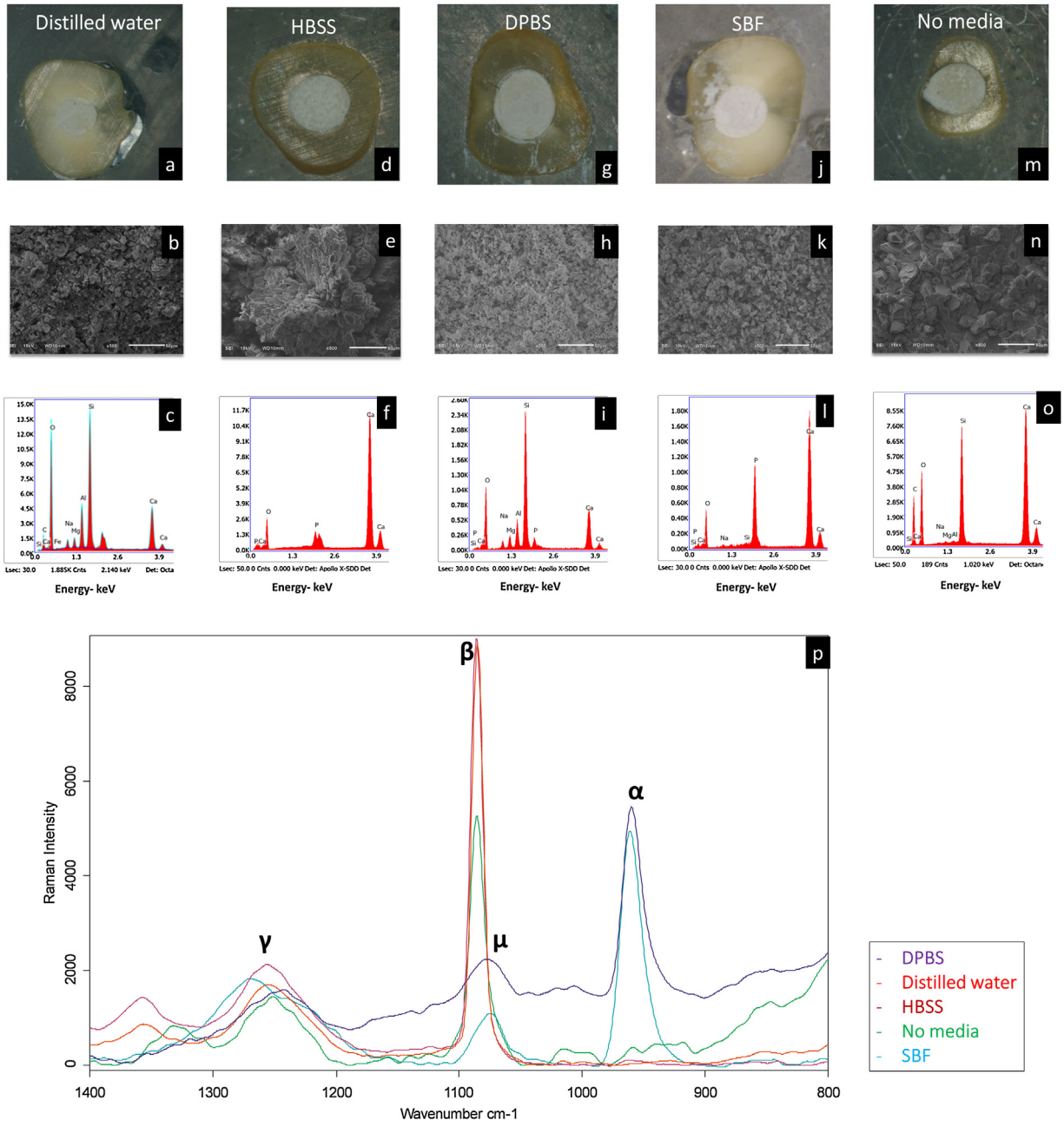

Fig. 3 – Analyses performed for Pure Portland Cement Med-PZ (Medcem, Weinfelden, Switzerland; MPC) stored in 5 different media. (a) Cylindrical dentin slice with a 2-mm thickness containing MPC in the center and stored for 1 month in distilled water; (b) SEM of MPC in (a) at 500×; (c) EDS of the MPC in (a); (d) Cylindrical dentin slice with a 2-mm thickness containing MPC in the center, stored for 1 month in Hank's balanced salt solution (HBSS); (e) SEM of the MPC in (d) 500×; (f) EDS of the MPC (d); (g) Cylindrical dentin slice with a 2-mm thickness containing MPC in the center and stored for 1 month in Dulbecco's phosphate-buffered saline (DPBS); (h) SEM of the MPC in (g) at 500×; (i) EDS of the MPC in (g); (j) Cylindrical dentin slice with a 2 mm thickness containing MPC in the center and stored for 1 month in simulated body fluid (SBF); (k) SEM of the MPC in (j) at 500×; (l) EDS of the MPC in (j); (m) Cylindrical dentin slice with a 2-mm thickness containing MPC in the center with no additional media; (n) SEM of the MPC in (m) at 500×; (o) EDS of the MPC in (m); (p) micro-Raman analysis for 5 different media with the following peaks: α: phosphate peak at 960 cm^{-1}, β: calcium-carbonate peak at 1082 cm^{-1}, γ: amide III band (N—H) at 1242 cm^{-1}, μ: peak for the carbonate group (β-type of carbonate) in hydroxyapatite at 1070 cm^{-1}.

[39]. Noticeable is that another study has reported a peak for β-type of carbonated apatite at 715–689 cm^{-1} that was not found throughout the current study [40].

Concerning the other MR vibrations recorded, the peak representing the amide III band (N—H) at 1242 cm^{-1} [27] was detected for the EV specimens in 5 different media (Fig. 3), indicating the presence of organic components (*e.g.* collagen) or

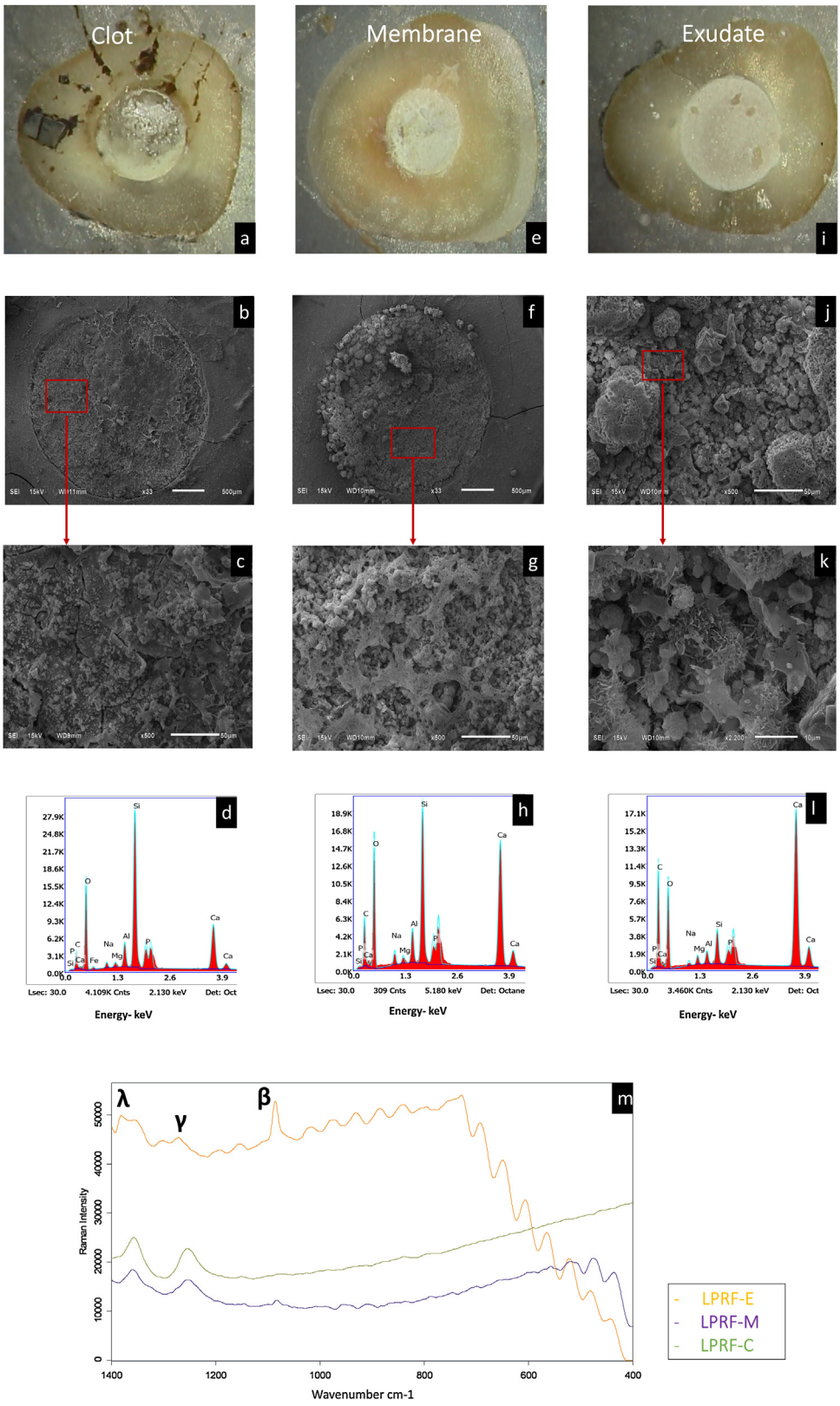

Fig. 4 – Analyses performed for Pure Portland Cement Med-PZ (Medcem, Weinfelden, Switzerland; MPC) in contact with leucocyte-and-platelet-rich fibrin (LPRF). (a) Cylindrical dentin slice with a 2-mm thickness containing MPC in the center, upon which a LPRF-clot (LPRF-C) was placed for 2 weeks; (b) SEM of the MPC in (a) at 33× (b) and at 500× (c); (d) EDS of the MPC in (a); (e) Cylindrical dentin slice with a 2-mm thickness containing MPC in the center, upon which a LPRF-membrane (LPRF-M) was placed for 2 weeks; (f) SEM of the MPC in (e) at 33× (f) and at 500× (g); (h) EDS of the MPC in (e); (i) Cylindrical dentin slice with a 2-mm thickness containing MPC in the center that was immersed in LPRF-exudate (LPRF-E) for 2 weeks;

bacterial contamination, even if SEM did not reveal any signs of bacterial growth. However, for the patient's sample and the LPRF specimens (Fig. 4), this amide III band was more obvious due to the direct contact with blood. The MR vibration for oxidized (ferric) proteins (Fig. 4m) and the detection of iron by EDS (Table 1) refer to the red blood cells in the thrombus of LPRF (Fig. 1).

Regarding the surface characteristics of MPC, except for the LPRF samples, none of the other samples were discolored. As the red thrombus of the LPRF-clot was in contact with MPC, a greyish discoloration occurred (Fig. 4a). The MPC-samples exposed to LPRF-membrane and -exudate were still yellowish discolored (Fig. 4e, i), even if there was no contact with the thrombus.

As the blood clot triggered during REP should reach approximately 3 mm below the cemento-enamel junction, a biocompatible material that is capable to induce mineralization, is required [1]. Nevertheless, this blood clot should be stable or a sterile and auto-resorbable collagen plug should be applied as a scaffold for TCS. If not, TCS might be pushed deeper into the root canal, as was the case in the present study. Hence, no further root development took place (Fig. 2a). Furthermore, the specimen retrieved from the patient (Fig. 2b) did not show the presence of calcium hydroxide but was rather rich in calcium carbonate and zinc phosphate due to contamination during REP with blood and zinc phosphate cement, respectively. Zinc phosphate was placed upon MPC as an intermediate layer between MPC and composite. However, the prescribed treatment protocols concerning REP rather recommend the application of GIC [1,41], even if research in materials have shown that the placement of GIC over fresh TCS cement interferes with cement setting [42]. The use of zinc phosphate was also representative in the weight percentage of the elements in MPC (Table 1): for the patient's sample the amount of zinc was prominent.

Even if *in vitro* settings strive to mimic as much as possible the clinical situation, it is questionable whether *in vitro* study results could be compared to clinical outcomes and *vice versa*. Before a biomaterial is applied in a clinical trial in order to predict its bioactivity, *in vitro* SBF-tests, *in vitro* cell culture tests and an *in vivo* animal study are performed. However, regardless the use of these pre-clinical tests, certain physiological and environmental conditions cannot be (easily) reproduced [20]. The lab protocol followed in this study might have influenced the material properties. Potential resin contamination due to embedding of the dentin slices, fixation and dehydration of MPC (= a non-living material), carbon-dioxide contamination during analysis and potential bacterial growth during storage in the incubator, may have occurred. Additionally, concerning the patient's REP-tooth in the current study, the restorative materials placed upon the MPC, the residual microbial load of the root canal post disinfection and the mechanical load of the tooth during function, are limitations to be aware of when interpreting the results. Additionally, patient-dependent factors such as general health, mouth hygiene and compliance during treatment, are not reproducible in lab conditions. Further research is necessary to distinguish the impact of these factors. Hence, caution is needed while interpreting *in vitro* results and extrapolating them to the clinical situation.

5. Conclusion

In the present study, only storage of MPC in DPBS and SBF for 1 month revealed apatite formation. Discrepancies between *in vitro* and clinical study protocols directly influence the outcome. Hence, the observed calcium carbonate was due to the presence of blood and/or environmental carbon dioxide. Furthermore, the detected amide III band indicated the presence of organic (collagen/blood) or bacterial components. In general, we can conclude that environmental conditions affect the bioactivity potential of MPC.

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(j) SEM of the MPC in (i) at 500× (j) and at 2200× (k); (l) EDS of the MPC in (i); (m) micro-Raman analysis for LPRF-E/M/C with the following peaks: β: calcium-carbonate peak at 1082 cm^{-1}, γ: amide III band (N—H) at 1242 cm^{-1}, λ: oxidized (ferric) proteins at 1378–1370 cm^{-1}.

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