Mineral deposition promoted by resin-based sealants with different calcium phosphate additions

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Abstract: The aim of this study was to evaluate the influence of different calcium phosphates (CaPs) on the physical, biological, and remineralizing properties of experimental resin-based sealants (RBSs). Triethylene-glycol dimethacrylate (90wt%) and bisphenol A-glycidyl methacrylate (10wt%) were used to produce resin-based sealants. Hydroxyapatite (S_{HAp}), α-tricalcium phosphate ($S\alpha_{-TCP}$) and octacalcium phosphate (S_{OCP}) were added to the sealants in a 10wt% concentration. One group without CaPs was used as the control group (S_{CG}). The degree of conversion (DC) was assessed with Fourier-transformed infrared spectroscopy, whereas cytotoxicity was tested with the HaCaT keratinocyte cell line. The ultimate tensile strength (UTS) was used to assess the mechanical strength of the experimental RBSs. Sealed enamel was used for colorimetric assay. Mineral deposition was assessed with Raman spectroscopy after 7, 14, and 28 days of sample immersion in artificial saliva. Scanning electron microscopy was used to analyze the surface morphology after 28 days of immersion. The addition of 10wt% of fillers significantly reduced the DC of sealants. S_{OCP} groups showed reduced cell viability. Higher UTS was found for $S\alpha_{-TCP}$ and S_{HAp}. The color analysis showed that S_{CG} and demineralized teeth presented higher mismatches with the sound tissue. Mineral deposition was observed for S_{HAp} and $S\alpha_{-TCP}$ after 7 days, with increased phosphate content and mineral deposits for S_{HAp} after 28 days. RBS with the addition of 10% HAp promoted increased mineralization *in vitro* after 28 days, and did not affect cell viability, DC, mechanical properties, or RBS color in the enamel.

Keywords: Dental Caries; Calcium Phosphates; Durapatite.

Introduction

Untreated dental caries remains a major problem in primary and permanent teeth.[1] Both noninvasive and microinvasive approaches have been applied to prevent caries progression and formation of cavities that may impact tooth aesthetics and function.[2,3] Microinvasive approaches, such as the sealing of pits and fissures, are effective in areas of the tooth that are more prone to caries development due to anatomic characteristics that make it more difficult to perform cleaning.[4,5] In these high-risk areas,

the use of sealants is a cost-effective method to prevent caries lesions and their progression.[6,7]

Sealing may be performed with different materials, such as glass ionomer cements or resin-based materials. Resin-based sealants (RBSs) have been used since the 1960s and are designed to penetrate inside deep regions of the tooth anatomy, and bond micromechanically to enamel, thus preventing demineralization in these regions.[8,9] Even though caries sealants are able to hamper the progression of initial caries lesions, the caries disease must also be controlled[10] with noninvasive strategies, such as oral hygiene education and healthy dietary practices. The success of these strategies is highly dependent on the patient's cooperation and use of sealants, especially in high-risk patients, in which case they may prevent against new caries lesions in surrounding areas.[7] The application of RBS alone is unable to stimulate remineralization at the resin-enamel interface to provide protection against further acid challenges.[11]

Calcium phosphates (CaPs) are inorganic compounds with structures and compositions similar to those of hard tissues of the human body.[12] These similarities have prompted studies on their use in regenerative medicine, as potential bioactive materials for application in various medical and dental treatments.[13] Although the ability of CaPs to promote mineral deposition depends on an increase in the calcium and phosphate contents of the medium, this mechanism is different for each CaP structure.[14] Hydroxyapatite (HAp) is the main mineral in the inorganic phase of the tooth and bone. Octacalcium phosphate (OCP) is known as a precursor of apatite, and has been studied as a rich calcium and phosphate source of mineralization, owing to its high solubility.[15] Among the tricalcium phosphates, alpha-tricalcium phosphate (α-TCP)

is used in bone cements due to its ability to form calcium-deficient HAp when dissolved in an aqueous medium. HAp, α-TCP, and OCP have already been studied in different applications in dentistry,[16–18] and the addition of CaPs to RBS may be an alternative to promote the remineralization of surrounding tissues and prevent further demineralization.[11] The aim of this study was to evaluate the influence of different CaPs on the physical, biological, and remineralizing properties of experimental resin-based sealants.

Methodology

Experimental enamel resin sealant preparation

The experimental sealants were prepared by mixing 90 wt% of triethylene-glycol dimethacrylate (TEGDMA), 10 wt% of bisphenol A-glycidyl methacrylate (BisGMA), 1 mol% of camphorquinone (CQ), and 1 mol% of ethyl 4-dimethylaminebenzoate (EDAB), all obtained from Sigma-Aldrich (St. Louis, USA). CaPs were added to 10wt% and separated into three experimental groups, namely HAp (S_{HAp}), α-TCP ($S\alpha_{-TCP}$), and OCP (S_{OCP}). One group without filler was used as the control group (S_{CG}). The characteristics of the CaP particles are described in Table 1. These particles were mixed with a polymeric blend, in an ultrasonic bath (L100; Schuster, Santa Maria, RS, Brazil), for eight minutes to ensure complete homogeneity of the sealants.

Degree of conversion

The degree of conversion (DC) of the experimental sealants was assessed with a Fourier-transformed infrared spectrophotometer (FT-IR Vertex 70; Bruker Optics, Ettlingen, Germany), equipped with an attenuated total reflection crystal (Platinum

Table 1. Characterization of tested calcium phosphates

Group	Chemical formula	Ca/p molar ratio	Particle size	Crystalline structure	Solubility at 25°C, g/l
HAp	$Ca_{10}(PO_4)_6(OH)_2$	1.67	26.8 nm	Hexagonal	0.0003[38]
α-TCP	α-$Ca_3(PO_4)_2$	1.50	6.03 μm	Monoclinic	0.002 [12]
OCP	$Ca_8(HPO_4)_2(PO_4)_4 \bullet _5H_2O$	1.33	4.94 μm	Triclinic	0.0081 [12]

ATR-QL; Bruker Optics, Ettlingen, Germany). The sealants were dispensed directly onto the diamond crystal (n = 3), in sections measuring 4 mm diameter and 2 mm thick. The spectra were taken before and after photocuring for 20 seconds, with 1,200 mW/cm² power (Radii Cal; SDI, Bristol, PA, USA). DC was calculated using the intensities of the aliphatic C=C peak at 1,637 cm⁻¹, compared with an internal standard carbonyl group C=O peak at 1,715 cm⁻¹ before and after light-curing.[19]

Cytotoxicity

Cell culture

The immortalized human keratinocyte HaCaT cell line was used (Banco de Células do Rio de Janeiro, Rio de Janeiro, Brazil) in this study. The cells were immersed in Dulbecco's modified Eagle medium (DMEM), complemented with 100 U/mL of penicillin, 100 µg/mL of streptomycin, 10% of fetal bovine serum, and 2 mM of L-glutamine. The cells were cultured at 37°C, under 5% CO_2/95% air atmosphere, and the medium was changed every two to three days.

SRB cytotoxicity assay

Eluates were prepared prior to cell treatment. Sealant specimens (4 mm in diameter and 1 mm in height; n = 3) were sterilized in an autoclave, and each was immersed in 1mL of DMEM for 24 hours at 37°C. The cells (5×10^3) were seeded in 96-well plates; after 24 hours, each well was treated with 100 µL of eluates added to the seeded wells. After 72 hours in culture, the cells were fixed in 50 µL of 50% trichloroacetic acid (Sigma-Aldrich, St. Louis, USA). Each well was covered with 50 µL of 0.4% sulforhodamine B (SRB) (Sigma-Aldrich, St. Louis, USA), as described previously.[20] The quantification of viable cells was performed for absorbance at 560 nm in a microplate spectrophotometer (Multiskan GO, Thermo Fisher Scientific, Waltham, USA), following the suspension of cells in 10% Trizma (Sigma-Aldrich, St. Louis, USA). Untreated medium was used as a control to normalize the percentage of viable cells.

Ultimate tensile strength

The ultimate tensile strength (UTS) analysis was performed with hourglass-shaped samples (n = 5) 8 mm long, 2 mm wide, and 1 mm thick, with a cross-sectional area of 1 mm² produced in a metallic matrix with a polyester strip at the bottom and top of the matrix. The samples were light-activated, applied for 20 seconds on each side, and stored in water for 24 hours at 37°C prior to testing. The UTS test was performed in a mechanical testing machine (Shimadzu EZ-SX; Shimadzu Corp., Kyoto, Japan) with a crosshead speed of 1 mm/min.

Mineral deposition

Mineral deposition was performed using micro-Raman spectroscopy (Senterra; Bruker Inc., Karlsruhe, Germany) equipped with a 100-mW diode laser adjusted to a 785-nm wavelength with a spectral resolution of ≈3.5 cm⁻¹ in three coadditions for five seconds. Specimens 4 mm in diameter and 2 mm high were produced for this analysis. Baseline measurements were taken after 24-hour immersion in distilled water. Then, one disc from each group was immersed in artificial saliva, prepared as described by Karlinsey et al.[24] Specimens were analyzed at 7, 14, and 28 days after immersion. The phosphate peak of 960 cm⁻¹ was used to measure the intensity of mineral deposits on the samples. After 28 days, the surface of the samples was scanned using scanning electron microscopy (SEM) (JSM 6060; JEOL Ltd., Tokyo, Japan), with the device set to a voltage of 3 kV.

Colorimetric assay

Thirty extracted bovine permanent incisors were stored in distilled water for 7 days after extraction. The samples were prepared by grounding flat the labial surface of the crowns, using silicon carbide sandpaper with grits of 600, 1,200 and 2,000 (Klingspor, Haiger, Germany), under water cooling. Then, the flat surfaces were polished (1-µm alumina suspension; Fortel, São Paulo, Brazil) in a polishing machine (Model 3v; Arotec, Cotia, Brazil) under water cooling for 60 seconds. Prior to demineralization, each enamel surface was partially covered with acid-resistant nail varnish, leaving an open window 5 mm × 5 mm.

Demineralization was created by subjecting the samples to an acid solution (pH: 4.4) produced as described previously.[21] The solution was maintained at 37°C for 72 hours, and the pH was measured twice a day using a pH meter (DM-22; Digimed, São Paulo, Brazil). Six teeth were used for each group to complete the colorimetric analysis. Six teeth were left with no demineralization, in order to conduct the analysis for sound enamel, and six remained unsealed to perform the demineralized enamel analysis. The demineralized enamel surfaces were etched with 37% phosphoric acid (Dentsply, York, USA) for 30 seconds, and rinsed with distilled water for the same amount of time. After air-drying, the experimental sealants were gently applied to the enamel surfaces with a microbrush (KG Sorensen; Cotia, Brazil) for 20 seconds, and light-cured for 20 seconds (Radii Cal; SDI, Bristol, USA). Color measurements were performed using a reflectance spectrophotometer (CARY 5000 UV-Vis-NIR; Agilent Technologies, Santa Clara, USA) equipped with a DRA-1800 integrating sphere. The color of each sample was measured and quantified in terms of three coordinate values (L*, a*, and b*), as established by the Commission Internationale de L'Eclairage (CIE). The color of the sound demineralized and sealed enamel samples was assessed under standardized conditions according to the CIE L*a*b* system. The differences among the parameters were used to calculate the ΔE, such as overall change in color, measured by comparing the mean ΔE of sound teeth with the ΔE of demineralized and sealed teeth.

Statistical analysis

Descriptive analysis of the specimens was performed for micro-Raman and SEM image analyses. The statistical analysis was performed using SigmaPlot 12.0 (Systat Software, San Jose, USA). The normality of data was evaluated using the Shapiro–Wilk test. The degree of conversion, the cytotoxicity, the UTS, and the colorimetric results were analyzed using one-way analysis of variance and Tukey's test.

Results

The DC values of RBSs (Table 2) ranged from 57.16% to 50.71%, with statistically significant higher values for S_{GC}, compared with CaP-containing sealants ($p < 0.05$; power: 81%). The percentages of cell viability are represented separately in Table 2. The S_{OCP} group presented statistically significant lower cell viability (82.82%; $p < 0.001$; power: 100%) than the S_{CG}, S_{HAp}, and $S\alpha_{-TCP}$ groups (97.12%, 95.88%, and 94.26%, respectively). UTS results are shown in Table 2. $S\alpha_{-TCP}$ presented statistically significant higher UTS values than S_{CG} and S_{OCP} ($p < 0.01$; power: 97%). Mineral deposition was found on the surface of samples containing α-TCP and HAp after 7 days of immersion in artificial saliva (Figures 1 and 2). A greater intensity in the phosphate peak (960 cm^{-1}) was observed in S_{HAp} after 28 days (Figure 2). SEM images showed that apatite-like particles were deposited on the surface of samples after 28 days of immersion (Figure 2). Colorimetric measurements are shown in Table 3. Higher ΔE values were found for demineralized teeth, compared with S_{OCP}, S_{TCP}, S_{HAp} values ($p < 0.05$; power: 84.4%). S_{GC} presented values similar to those of demineralized and sealed teeth. Finally, L* values were statistically higher for demineralized teeth than sound sealed surfaces ($p < 0.05$; power: 99%).

Table 2. Degree of conversion (%), cell viability (%) and ultimate tensile strength means and standard deviations.

Group	Degree of conversion (%)	cell viability (%)	Ultimate tensile strength (MPa)
S_{CG}	57.16 ± 1.85 A	97.12 ± 3.35 A	22.64± 6.88 C
S_{OCP}	50.71 ± 1.57 B	82.82 ± 1.95 B	23.78 ± 5.97 BC
$S_{\alpha-TCP}$	51.84 ± 1.70 B	94.24 ± 1.62 A	35.44 ± 6.72 A
S_{HAp}	52.61 ± 1.51 B	95.87 ± 0.50 A	30.99 ± 5.40 AB

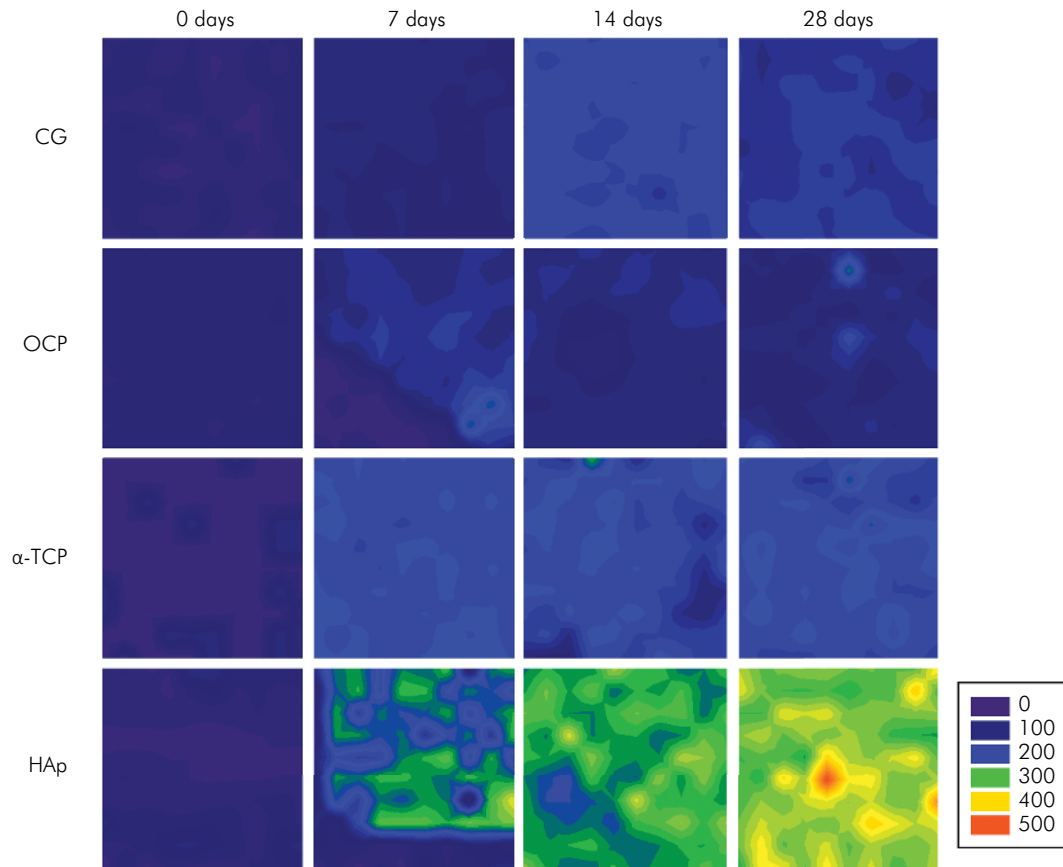Different uppercase letters indicate statistical difference among groups.

Figure 1. Color representation of the intensity of phosphate peaks (960 cm^{-1}) in the different tested groups after 7, 14 and 28 days of immersion in saliva. Higher intensities are represented by red/green. Blue regions represent lower or total absence of phosphate intensity.

Different lowercase letter indicates a statistical difference in the same row ($p < 0.05$); Different uppercase letter indicates a statistical difference in the same column ($p < 0.05$).

Discussion

CaPs have been widely studied due to their potential to induce mineral deposition.[13] In this study, three different calcium phosphates (HAp, OCP, and α-TCP) were added to a resin-based sealant. The DC was influenced by the addition of HAp and α-TCP, whereas lower cell viability was found for OCP. Mineral deposition was observed for the α-TCP and HAp samples after 7 days of immersion in saliva. After 28 days, Raman analysis and SEM images confirmed a higher rate of mineral deposition in the S$_{HAp}$ group.

The addition of fillers to a polymeric blend may lead to a different light-scattering pattern in the composite, resulting in differences in the number of converted monomers in the composite.[22,23] All CaP-containing groups presented lower DC values than the control group (Table 2). The addition of filler resulted in increased opacity of the experimental sealants, and this could promote a reduction in light passage during polymerization.[24] Although a statistically significant difference was found, the values obtained were similar to those for commercial sealants.[25] Differences in DC may lead to reduced mechanical properties and increased cytotoxicity.[26] Conversely, in the UTS analysis, the RBSs with HAp and α-TCP additions presented higher tensile strengths than the sealants without filler (S$_{GC}$; Table 2). Sealants must have adequate mechanical properties, since they are frequently placed in areas subjected to masticatory

forces, such as occlusal areas. The addition of fillers to resin-based materials may increase their tensile strength,[27] and this phenomenon could have occurred with the addition of HAp and α-TCP, considering that their crystalline structure may be responsible
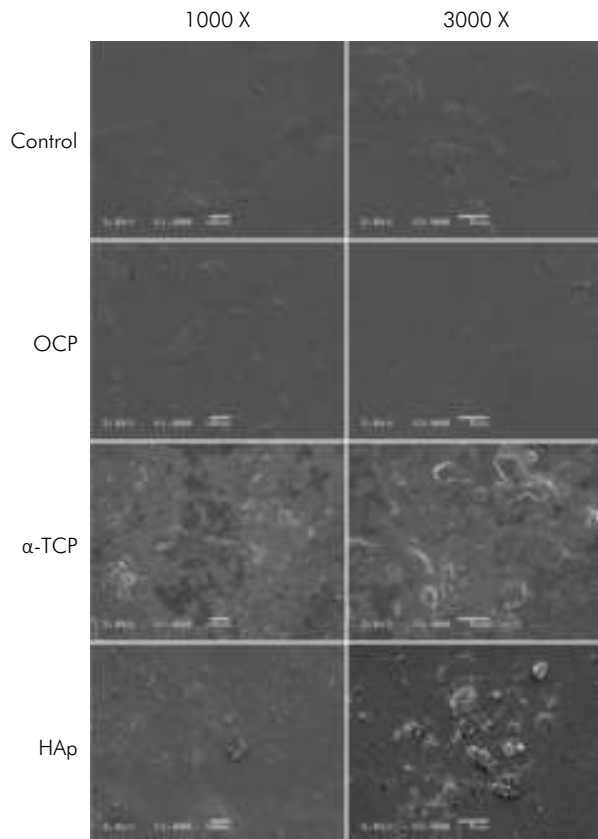

Figure 2. SEM images (1,000× and 3,000×) of samples after immersion in saliva for 28 days. Mineral deposits are observed for the HAp and α-TCP groups and are better observed at 3,000× magnification. OCP did not promote deposition in the specimens.

for higher mechanical strength, in comparison with the less packed structure of OCP, as shown in Table 1.

The experimental sealants were produced with high TEGDMA content, a formulation chosen to adjust the viscosity of the RBSs and facilitate penetration of materials in the pits and fissures. However, TEGDMA is known to be the most leachable monomer among the dental composites, and its release may cause cell death.[26] The release of unreacted monomers may affect gingival tissues, and human keratinocytes were used in this study as an epithelial cell line to assess the influence of materials on cell behavior. Higher viability was observed for RBSs containing HAp and α-TCP , but there was no statistically significant difference compared with the control group (Table 2). Since OCP is the most soluble[13] filler, and its release may influence polymeric matrix stability, other unreacted monomers may be leached from the TEGDMA/BisGMA blend. Higher hydrolytic stability of the fillers in S_{HAp} and $S\alpha_{-TCP}$ probably leads to higher cell viability.

The addition of bioactive fillers may affect the color of RBSs, and be inadequate if placed in aesthetic areas. In the present study, demineralized enamel was sealed with different CaP-containing sealants, the colors of which were analyzed with the CIE L*a*b* system. The application of different colorimetric measurements, such as the CIE L*a*b* and the CIED2000, results in variations in visual perception and tooth aesthetics.[33] Although CIED2000 is regarded as representing better visual perception in dentistry,[34] the CIE L*a*b* parameters may be effective in detecting differences between demineralized and sound enamel before and after sealing procedures. Higher values of ΔE were observed for S_{GC} and demineralized surfaces,

Table 3. Colorimetric analysis of sound, demineralized and sealed enamel. L*a*b parameters and ΔE calculated for sealants in sound and demineralized enamel.

Group	Sound (ΔE)	Demineralized (ΔE)	L*	a*	b*
Sound	-	-	88.65 ± 0.97ᵃ	-0.43 ± 0.09ᵃ	-0.51± 0.95 ᵃ
Demineralized	-	-	93.22 ± 1.61ᵇ	-0.76 ± 0.12 ᵃ	-2.13 ± 0.39ᵇ
s_{CG}	1.32 ± 0.91 ᴬᵃ	4.14 ± 1.91 ᴬᵇ	89.31 ± 0.62ᵃ	-0.12 ± 0.14 ᵃ	-0.66± 0.41 ᵃ
s_{OCP}	1.22 ± 0.71 ᴬᵃ	4.77 ± 1.63 ᴬᵇ	88.43 ± 0.66ᵃ	-0.26 ± 0.12 ᵃ	-0.66 ± 0.76 ᵃ
$s_{\alpha-TCP}$	1.51 ± 0.40 ᴬᵃ	5.03 ± 1.20 ᴬᵇ	88.64 ± 0.55ᵃ	-0.08 ± 0.15 ᵃ	-0.22 ± 0.57 ᵃ
S_{HAp}	1.60 ± 0.27 ᴬᵃ	4.80 ± 1.39 ᴬᵇ	88.75 ± 0.43ᵃ	-0.06 ± 0.13 ᵃ	-0.02 ± 0.37 ᵃ

indicating that CaP-containing groups attained a color value closer to that of sound tissue (Table 3). In addition to the overall color difference (ΔE), the lightness—assessed as the L* value—is an important parameter to be considered, since an increase in the L* parameter may indicate higher demineralization in early caries lesions. [35] The light-scattering that occurs through poorly mineralized enamel increases L* values closer to 100, which represents the white color balance. Application of sealants to demineralized enamel leads to reduced L* and lightness values for sound enamel, thus indicating that light-scattering in demineralized enamel was improved by the sealing procedure.

Remineralization in already sealed areas may help avoid the formation of new caries lesions, especially in high-risk patients.[7] The addition of HAp and α-TCP to resin sealants promoted *in vitro* mineral deposition, especially in S_{HAp} after 28 days of immersion in artificial saliva. The CaP mechanism to promote mineral deposition has been studied extensively to date[29,36], and depends on increasingly higher concentrations of Ca^{2+} and PO_4^{3-} in the medium. The availability of these ions promotes greater apatite nucleation and mineralization of hard tissues.[13] Differences in mineral deposition can be explained by variations in CaP structures (Table 1). OCP has the highest solubility among the tested CaPs, owing to its low crystal arrangement and Ca/PO_4 ratio. Although the triclinic structure of OCP is similar to the hexagonal HAp structure, the presence of a hydrated sublayer in the less densely packed calcium and phosphate atoms[30] is responsible for the high solubility of OCP. It could be expected that the high solubility of OCP would increase Ca^{2+} and PO_4^{3-} availability; however, quick loss of these ions prevents the nucleation of apatite crystals, thus explaining the lower mineral formation observed in the present study. The dissolution process may be why the phosphate content was not as high for $S_{α-TCP}$ as S_{HAp}. α-TCP has a lower Ca/P ratio compared with HAp, leading to a reduced level of stability.[32] The α-TCP used in the present study had the same composition as β-TCP, the most frequently used tricalcium phosphate; however, they differ in their crystal structure, which makes α-TCP more soluble than β-TCP. In the present study, monoclinic α-TCP was used due to its ability to create calcium-deficient HAp upon hydrolyzation,[12]

thus promoting a nucleation site for mineral deposition. Even though other authors have reported the CaP mechanism,[12] the mineral deposition of $S_{α-TCP}$ was lower when compared to that of S_{HAp}.

In vivo remineralization is a complex process, and mineral deposition *in vitro* is one way to assess the ability of a material to induce phosphate formation on the surface of test samples. In this study, the S_{HAp} group showed increased phosphate content (960 cm^{-1} peak; Figure 1) and mineral deposition after 28 days (Figure 2), compared with the other experimental groups. Although it is not possible to extrapolate these *in vitro* results directly, some studies have suggested that HAp may induce apatite nucleation in tooth and bone.[37,38] HAp is known as the least soluble CaP, and the stability of the particles is responsible for controlled Ca^{2+} and PO_4^{3-} release, and later nucleation of mineral deposits.[22] Nano-sized HAp was used in the present study, whereas OCP and α-TCP were of micrometric size (Table 1). The different particle sizes used represent a limitation of the present study, since fillers were added by wt%; therefore, S_{HAp} received a higher volume of particles. Nano-sized HAp has already been synthesized using different methods,[39,40] whereas little information can be found on the subject of nanometric OCP and α-TCP. [41] The nanometric size of HAp increased the surface area of the particle, thereby increasing the area for calcium phosphate nucleation on the surface of the samples, according to the outcome observed by mineral deposition analysis.

The addition of HAp to enamel resin sealants promoted an increase in the mineral deposition after 28 days of immersion in saliva, and enhanced the potential therapeutic effect of these materials. No cytotoxic effect and adequate mechanical properties were found for S_{HAp}. The more soluble α-TCP and OCP fillers showed little or no mineral deposition, and may not be suitable for the remineralization of pits and fissures and surrounding enamel.

Conclusion

Resin-based sealants with the addition of 10% HAp promoted increased mineralization *in vitro* after 28 days, and did not impact cell viability, mechanical properties, or RBS color in the enamel.

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