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In vitro apatite-forming ability of calcium aluminate blends

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ABSTRACT

Calcium aluminate cement (CAC) blends show great potential as biomaterial when compared to commercial products used in odontology and orthopedics. Mixtures of CAC +4 wt% of different additives (alumina, zirconia, zinc oxide, tricalcium phosphate or hydroxyapatite) containing compositions, resulted in samples with low porosity levels and smaller pore sizes after their contact with simulated body fluid (SBF) solution, which was associated with apatite precipitation on the materials' surface. In order to certify these aspects, the in vitro apatite-formation ability (bioactivity) of CAC blends was evaluated by pH and calcium concentration measurements in SBF for samples previously treated (or not) with sodium silicate (SS) solution. The surface of the samples after immersion in SBF or SBF/SS was analyzed by scanning electron microscopy (SEM), energy dispersive X-ray (EDX) analysis and confocal Raman spectroscopy. In addition, the in vitro apatite deposition and the osteoblastic cell viability were also evaluated. SEM results showed that the precipitation of phases was detected on the CAC blend samples' surfaces. The presence of calcium and mainly phosphorus by EDX indicated the formation of calcium phosphate phases. Moreover, the presence of a more homogeneous apatite-like layer on the samples' surface was observed after treatment with sodium silicate solution. The detection of the Raman signature at 960 cm^{-1}, confirmed the presence of an apatite-like layer on the surface of the compositions after immersion in SBF or SBF/SS. Regarding the osteoblastic cell viability results, blends with collagen, zinc oxide and zirconia presented better results when compared to commercial products.

1. Introduction

Calcium aluminate (Ca-aluminate) based materials show great potential in the biomaterial area due to their unique curing/hardening characteristics and related microstructure. An overview regarding Ca-aluminate applications in odontology and orthopedics has been already published [1].

Calcium aluminate cement (CAC) presented a suitable performance as a root-end filling component [2,3], overcoming some drawbacks of commercial calcium silicate-based compositions and MTA (mineral trioxide aggregate), such as: long setting time, high porosity and low mechanical strength. CAC also shows interesting features as a biomaterial, for example: *(i)* it can provide better flowability and handling properties [4,5], *(ii)* adjustable rheology and setting time at room temperature, resulting in high initial mechanical strength [6], *(iii)* biocompatibility without inflammatory reactions when tested in subcutaneous tissues of rats [7,8], *(iv)* it can act as a barrier preventing bacterial microleakage [9], and *(v)* present low expansion, which favors good retention and adhesion to the teeth [6].

Some studies have also reported CAC applications for repairing bone defects, based on the fact that its chemical composition and thermal expansion coefficient are similar to teeth and human bones [3,10]. CAC also has the potential to be used in orthopedics, as it can avoid some problems of commercial products (PMMA, polymethyl methacrylate) regarding its handling behavior (too low consistency and strong odor) and exothermic reactions (local heat increase which can damage the surrounding tissue).

A previous study by some of the present authors [11] evaluated the properties of various CAC-based mixtures containing different amounts of various compounds (alumina, zirconia, zinc oxide, hydroxyapatite, tricalcium phosphate, chitosan or collagen) and compared them with commercial products used in dentistry (MTA and glass ionomers) and orthopedics (PMMA). The attained results showed that plain CAC resulted in suitable compressive strength. Besides that, the mixtures of CAC +4 wt% of different compounds (alumina, zirconia, zinc oxide, tricalcium phosphate or hydroxyapatite) resulted in samples with

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similar or higher mechanical strength than PMMA. On the other hand, adding chitosan or collagen to the CAC (mainly above 2 wt%) increased the porosity and reduced the mechanical resistance to CAC under compression due to the higher average particle size of these additives.

However, compositions containing 4 wt% of alumina, zirconia, zinc oxide, tricalcium phosphate or hydroxyapatite or 1 wt% of chitosan or collagen, presented lower porosity and narrower pore size distribution after their interaction with simulated body fluids (SBF). This behavior was most likely associated to apatite formation on the materials' surfaces, as the precipitated phosphates (derived from the interaction of the cements with the compounds available in the SBF solution) might coat the solid surface and fill in the pores. Among the commercial products evaluated, only MTA showed similar behavior [11].

Considering these aspects, this paper focuses on evaluating the bioactivity of these compositions. One common method that has been used for testing in vitro bioactivity is to quantify the apatite-formation ability in SBF [12,13]. Detecting apatite on the surface of a material immersed in SBF is useful for predicting its in vivo bone bioactivity. Besides that, it can help to reduce the number of animals used for testing and the length of time of the experiments, speeding up the development of new types of bioactive materials [12].

According to the literature, the bioactivity has been usually attributed to the ability to generate hydroxyapatite in the presence of phosphate-containing solution [14,15]. The resulting surface hydroxyapatite layer is similar in structure to the mineral components of bone and teeth [16]. Materials with this surface layer develop chemical bonds and biological integration with the bone [17]. More recently, bioactivity was defined as the material's ability to generate a carbonated hydroxyapatite layer on its surface in order to induce strong interfacial bonds with living tissues [18].

Bioactivity is also an important aspect, as it provides better bonding between the dental materials and the tooth structure and may help to prevent the formation of secondary caries (derived from remaining voids between the filling material and the teeth, allowing the migration of bacteria to these regions) and consequently the replacement of the dental restoration. In addition, apatite formation in situ and strong bonding are parameters that make dental restoration closer to the original structure, which are desirable characteristics, especially when compared to other fillers such as amalgam.

However, for a thorough evaluation of the in vitro bioactivity, it is recommended to combine the tests in SBF with experiment methods using cells [13,19]. The ability to stimulate a cell response is very important and complements the in vitro apatite deposition results, which is also presented in this paper.

2. Materials and techniques

The compositions in this study were prepared by dry-ball-mixing for 1 h calcium aluminate cement (Kerneos Aluminates, France [5]) with a polyglycol-based dispersant (0.6 wt%, Basf, Germany) and a plasticizer $CaCl_2{\cdot}2H_2O$ (2.8 wt%, Labsynth, Brazil). The following high purity compounds were incorporated into the general composition, called CACH: (1) calcined alumina (CT3000SG, Almatis, USA), (2) monoclinic zirconia (CC-10, Saint-Gobain, France), (3) zinc oxide (Synth, Brazil), (4) hydroxyapatite (Sigma-Aldrich 21223, USA), (5) tricalcium phosphate (Cadisa, Brazil), (6) chitosan (Polymar, Brazil) or (7) bovine collagen (type I, JBS, Brazil). Additionally, commercial products were analyzed as reference materials: dentistry cements, such as white MTA (Angelus, Brazil), glass ionomers for cementing and restoration (Meron, Voco, Germany) and for base and lining (Vidrion F, SS White, Brazil), and PMMA cement (Bio mecânica, Brazil).

A total of 4 wt% of alumina, zirconia, zinc oxide, hydroxyapatite or tricalcium phosphate, or 1 wt% of chitosan or collagen, was added to CACH and these compositions were dry-ball-mixed for 1 h. After that, aqueous suspensions (80 wt% of solids) were prepared using a lab mixer and cylindrical samples were molded (diameter =11 mm and height =4 mm) for pH and calcium concentration measurements. Another set of these samples (diameter =10 mm and height =1 mm) were prepared to analyze the apatite-forming ability on their surfaces. Collagen containing compositions required extra water (73 wt% of solids) to be molded.

The samples were kept at 37 °C for 24 h in a water saturated environment, demolded and dried at 37 °C for another 24 h. After that, they were placed into plastic containers with 33 mL of simulated body fluid (SBF) solutions and kept at 37 °C for 21 days. These solutions had their pH and calcium concentration measured at certain time intervals, using a pH sensor connected to an automatic data recorder system (MA 522/E, Marconi, Piracicaba, Brazil) and a selective sensor for calcium ions (series 930, Aprolab, Brazil).

The samples were also placed into plastic containers with 19 mL of SBF solutions to maintain a surface area-volume ratio of 0.1 cm^{-1} and kept for 21 days under stirring at 37 °C using a shaker (MA420, Marconi). Afterwards the samples were gently rinsed with deionized water, followed by drying at room temperature according to the literature [20]. The surface of the samples was analyzed by scanning electron microscopy (SEM, EVO MA10 Zeiss), energy dispersive X-ray analysis (EDX) and confocal Raman spectroscopy (Rivers diagnostic system, model 3510) coupled to a red laser with a wavelength of 785 nm. The same tests described above were also carried out using samples previously treated for 7 days with sodium silicate (SS) solution (33 or 19 mL depending of sample size) and using the commercial products selected as a reference. In the latter case, the samples were prepared according to the instructions provided by the manufacturers.

Aqueous CACH suspensions containing 4 wt% of additives (alumina, zirconia, zinc oxide, hydroxyapatite or tricalcium phosphate) or 1 wt% of chitosan or collagen, were also prepared, molded as cylindrical samples (diameter =6 mm and height =2 mm) and demolded under sterile conditions (laminar flow cabinet) and cured at 37 °C for 24 h in a saturated environment (relative humidity =100%). After that, the samples were dry-sterilized at 110 °C for 24 h, followed by exposure to an ultraviolet germicidal lamp for 20 min. Samples of commercial products were also prepared and sterilized for cell culture experiments and for alkaline phosphatase (ALP) activity evaluation.

2.1. Cell culture

Osteoblastic cells derived from human osteosarcoma (SAOS-2, ATCC HTB-85) were grown as monolayer cultures in T-75 flasks (Corning Inc., NY, EUA) containing 10 mL of McCoy´s 5 A medium (Sigma St Louis, MO, EUA), which was enriched with 15% fetal bovine serum (Invitrogen, Carlsbad, CA, EUA), 100 μg/mL of streptomycin and 100 UI/mL of penicillin (Gibco, Gran Island, NY, EUA). This enriched medium was kept at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% of atmospheric air. After confluence (a condition where a suitable number of adherent cells in a culture dish is obtained), the cells were detached with ethylenediaminetetraacetic acid (EDTA solution −1 mM, Gibco) and trypsin (0.25%, Gibco) for 2 min. A fixed quantity of cells (2×10^4) was placed on a well in triplicate, using a culture dish with 24-wells. The cell culture was cultivated for 24 h in osteogenic medium enriched with 7 mM of β-glicerofosfato (Sigma) and 50 μg/mL of ascorbic acid (Sigma).

Samples were placed on polycarbonate membrane inserts (pore size 3 μm, Greiner Transwell) and cell exposure was carried out for periods up to 7 days. Non-exposed cultures and those exposed to aqueous medium containing 10 vol% of dimethyl sulfoxide (DMSO) were used as positive and negative controls, respectively.

2.2. Cell viability test (MTT)

On day 1 after the samples exposure to the cell culture, the cell viability test (which evaluates the ability of cells to maintain or recover