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ScienceDirect
Materials Science and Engineering C 28 (2008) 347–352
MATERIALS SCIENCE & ENGINEERING C
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Bioactive coatings on Portland cement substrates: Surface precipitation of apatite-like crystals

Daniel Gallego [a], Natalia Higuita [a,b], Felipe Garcia [b], Nicholas Ferrell [a], Derek J. Hansford [a,*]

[a] *Biomedical Engineering Department, The Ohio State University, 1080 Carmack Road, 270 Bevis Hall, Columbus (OH) - 43210, United States*
[b] *Grupo de Investigación en Ingeniería Biomédica CES-EIA (GIBEC), Carrera 43 A No. 52 Sur – 99, Sabaneta, Colombia*

Available online 14 April 2007

Abstract

We report a method for depositing bioactive coatings onto cement materials for bone tissue engineering applications. White Portland cement substrates were hydrated under a 20% CO_2 atmosphere, allowing the formation of $CaCO_3$. The substrates were incubated in a calcium phosphate solution for 1, 3, and 6 days (CPI, CPII, and CPIII respectively) at 37 °C to induce the formation of carbonated apatite. Cement controls were prepared and hydrated with and without CO_2 atmosphere (C+ and C− respectively). The presence of apatite-like crystals was verified by Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDS). The substrate cytocompatibility was evaluated via SEM after 24 hour cell cultures. SEM revealed the presence $Ca(OH)_2$ on C−, and $CaCO_3$ on C+. Apatite-like crystals were detected only on CPIII, confirmed by phosphorus EDS peaks only for CPIII. Cells attached and proliferated similarly well on all the substrates except C−. These results prove the feasibility of obtaining biocompatible and bioactive coatings on Portland cement for bone tissue engineering applications.

Keywords: Portland cement; Carbonated apatite; Bioactivity; Bone tissue engineering; Calcium phosphates

1. Introduction

There is a constant need for bone substitutes due to severe bone injuries, degenerative diseases, and certain cases of reconstructive surgery [1]. Traditional treatments such as autografts and allografts present several disadvantages due to donor site morbidity, limitations on the size of bone that can be harvested, and the potential for immune rejection and infections [2,3].

Bone tissue engineering (BTE) seeks to promote the regeneration (*in vivo* or *in vitro*) of damaged or lost bone tissue through therapies based on a combination of scaffolds, growth factors, and cells [4]. Scaffolds are either temporary or permanent matrices usually made of synthetic or naturally occurring materials, responsible for supporting the growth of new tissue; however, most of these materials are still far from completely fulfilling both the necessary biological and mechanical functions in order to perform successfully as bone substitutes [5].

In addition to biocompatibility, load bearing capabilities, and an interconnected structure with a defined pore size, BTE scaffolds are also required to exhibit a bioactive behavior, which

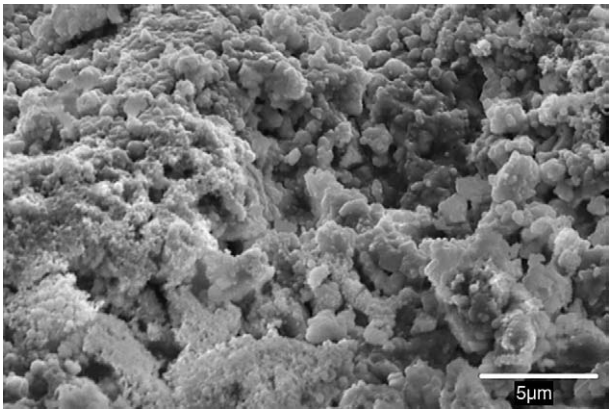

Fig. 1. SEM image of a Portland cement substrate (C−), exhibiting the typical granular microstructure of this material.

* Corresponding author.
E-mail address: hansford.4@osu.edu (D.J. Hansford).

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doi:10.1016/j.msec.2007.04.020

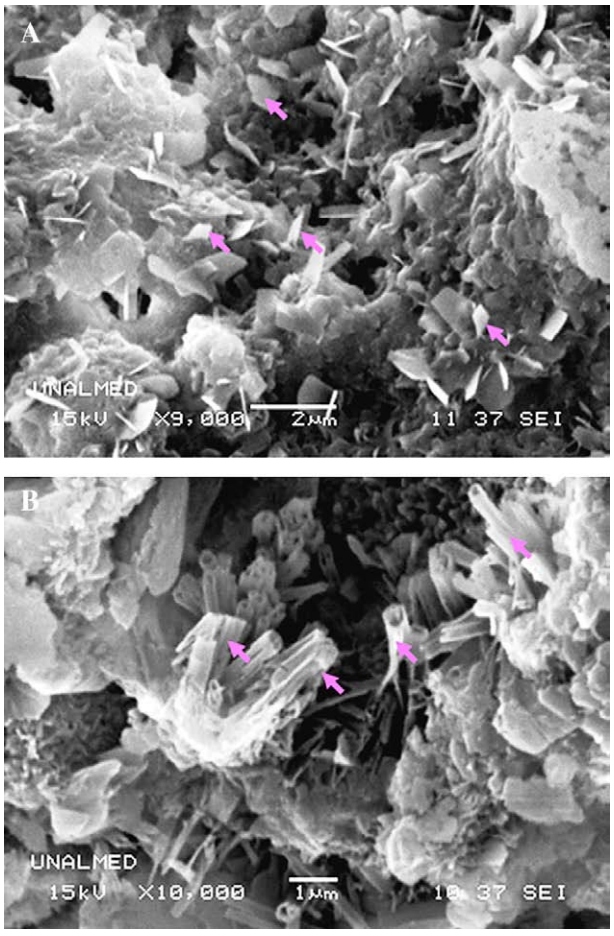

Fig. 2. A. High magnification SEM image of C− showing $Ca(OH)_2$ crystals (purple arrows). B. High magnification SEM image of C+ exhibiting $CaCO_3$ crystals (purple arrows) (for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

means they should promote a strong osteointegration, and stimulate the growth of bone cells [6–10].

For bioactivity purposes several calcium phosphates such as hydroxyapatite, tetracalcium phosphate, and tricalcium phosphate, among others, have been widely investigated because they present similarities to the mineral phase of native bone tissue [11].

Carbonated apatite (CA) is a type of apatite with carbonate ion (CO_3^{-2}) substitutions in either phosphate (PO_4^{-3}) or hydroxyl sites ($(OH)^{-1}$). CA has a higher similarity with the mineral phase in bone due to its low crystalline order and small crystal size as compared to pure hydroxyapatite; such characteristics seem to provide superior osteogenic properties because of its higher solubility and resemblance to the natural tissue [12].

Previous research has demonstrated the potential uses of modified Portland cement for BTE applications due to its biocompatibility and load bearing capabilities [13–15]. This work is focused on developing a methodology for producing apatite-like phases in the cement to increase the bioactivity of this material.

2. Materials and methods

2.1. Substrate fabrication and characterization

White Type I Portland cement (Cementos Argos, Colombia) was mixed with distilled water at a 2:1 ratio (wt/wt). The mixture was allowed to settle and harden in rubber molds (2 cm in diameter, 0.5 cm thick) for 2 h. The cement substrates were removed from the molds and hydrated in an incubator for 8 days (20% CO_2, 37 °C, and 90% relative humidity). A group of control substrates was fabricated under the same conditions, but the hydration was not done under a CO_2 atmosphere (C−).

For the bioactive coatings, a calcium phosphate solution was prepared by diluting $CaCl_2$ (Merck, USA) in phosphate-buffered saline (ATCC, USA) at a final concentration of 2 M [16]. The CO_2 treated substrates were exposed to this solution for 1 day (CPI), 3 days (CPII), and 6 days (CPIII) at 37 °C. Substrates that were treated with CO_2 during hydration but were not exposed to the calcium phosphate solution served as carbonated controls (C+).

The samples (CPI, CPII, CPIII, C+, and C−) were washed twice with deionized water and dried in an oven at 100 °C for 24 h. These were then prepared for SEM and EDS characterization by sputter coating with a thin layer of gold/palladium.

2.2. Cytocompatibility experiments

Human osteosarcoma cells, HOS, (ATCC, USA) were seeded on top of previously sterilized (by autoclaving) substrates (CPI, CPII, CPIII, C+, and C−) at a density of 5×10^4 cells/cm^2, and cultured for 24 h using Minimum Essential Medium (ATCC, USA) supplemented with 10% FBS (ATCC, USA) and 1%

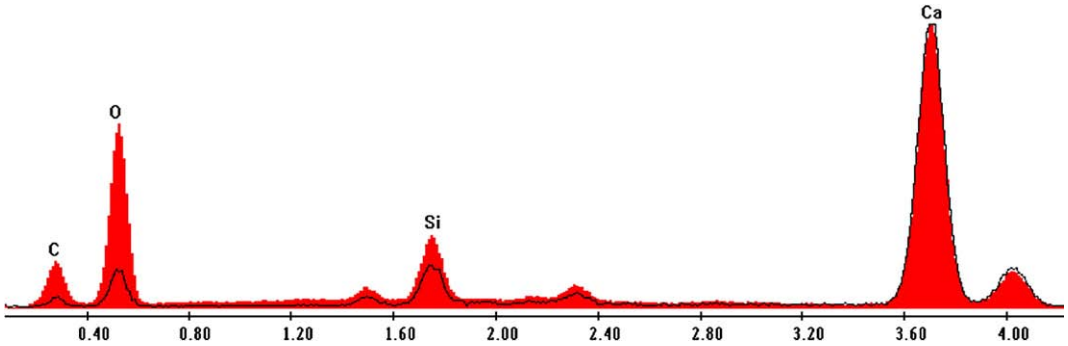

Fig. 3. Comparison of the EDS spectra for C+ (red) and C− (black) (for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

antibiotics (ATCC, USA) in a humidified incubator (5% CO_2, and 37 °C).

After incubation, the cells were fixed with 70% ethanol at − 20 °C for 30 min, then dehydrated in graded ethanol solutions (70, 80, 90, and 100%) and hexamethyldisilazane (Ted Pella, USA) according to the procedure described by Braet et al. [17].

3. Results

3.1. Substrate fabrication

With the described methodology, it was possible to produce hardened Portland cement substrates 2 cm in diameter and approximately 5 mm thick. Under SEM imaging the typical granular microstructure of hydrated Portland cement was observed (Fig. 1).

SEM characterization revealed the presence of calcium hydroxide (CaO_2H_2) crystals in C−, while C+ showed mostly calcium carbonate ($CaCO_3$) crystals (Fig. 2) in addition to the typical calcium silicate hydrate (CSH) gel found in hydrated Portland cement pastes. EDS analysis confirmed a high intensity peak of carbon for C+ (Fig. 3).

CPIII was the only substrate that exhibited the formation of apatite-like crystals on the surface (Fig. 4) after incubation in the calcium phosphate solution; such crystals were not detected on any of the other samples (C−, C+, CPI, and CPII). EDS characterization confirmed phosphorous peaks only for CPIII (Fig. 5). Elemental mapping showed that the crystals had a high concentration of carbon and oxygen as well (Fig. 6).

Exposure of C− to the calcium phosphate solution under the same conditions was run in parallel experiments (data not shown). SEM inspection revealed that no apatite-like crystals were precipitated on these samples after incubation (at either 1, 3, or 6 days). This was further confirmed by lack of phosphorous peaks in the EDS spectra.

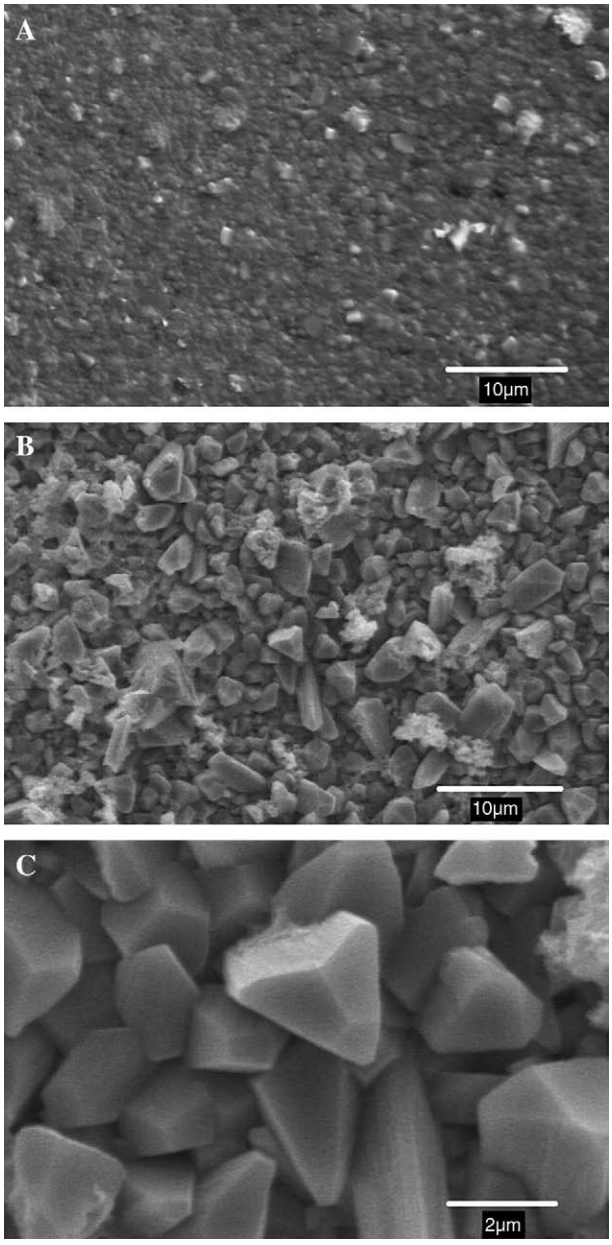

Fig. 4. A. SEM micrograph of C+ showing no crystals on the surface. B and C. SEM micrograph (two different magnifications) of the apatite-like crystals precipitated on CPIII.

3.2. Cytocompatibility experiments

Cell adhesion, spreading and proliferation was observed to be normal on C+, CPI, CPII, and CPIII (Fig. 7B, C). After 24 h of culture, the cells formed a nearly confluent monolayer on the surface of the substrates. No cells were able to adhere and proliferate on C− (Fig. 7A). The cells that were cultured on CPIII seemed to be more flattened (Fig. 8A) compared to the cells that were cultured on C+, which appeared to have more individual cytoplasmic extensions (Fig. 8B).

4. Discussion

Portland cement is mainly composed of calcium silicates (tricalcium silicate, C_3S, and dicalcium silicate, C_2S), which react with water during the hydration and produce calcium silicate hydrate and calcium hydroxide, providing high strength and alkalinity to the material, respectively [18].

SEM micrographs showed different types of crystals for C+ and C−. This was due to the introduction of CO_2 throughout the hydration process for C+, which triggered a carbonation reaction, thus converting the $Ca(OH)_2$ into $CaCO_3$ and releasing water (reaction (1)) [19]. The EDS peak for carbon in C+ was noticeably higher than the one in C−. The small peak of carbon presented in C− could be attributed to carbonation caused by atmospheric CO_2 [20].

$$Ca(OH)_2 + CO_2 \rightarrow CaCO_3 + H_2O \quad (1)$$

Apatite-like phases were observed on the carbonated substrates (C+) only after 6 days of incubation with the calcium phosphate solution (CPIII). The crystals covered a noticeable surface area but did not coat the samples entirely. Wang et al. [21]

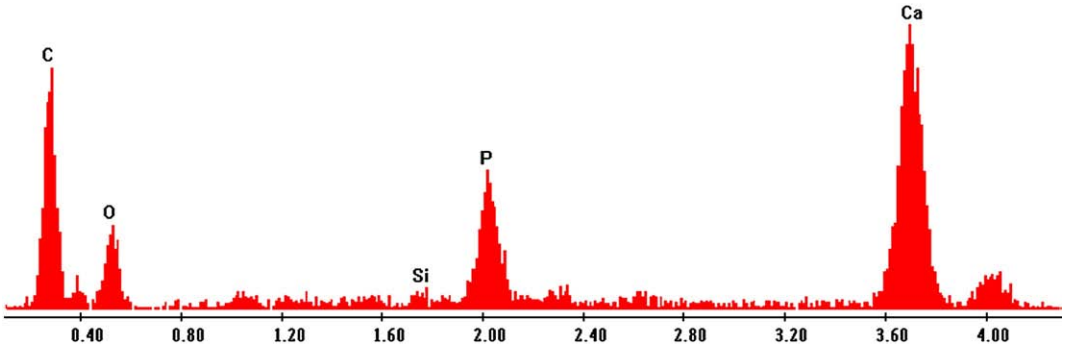

Fig. 5. EDS spectrum for CPIII.

reported the precipitation of morphologically similar crystals on the surface of hydroxyapatite substrates after 15 days of exposure to a phosphate-buffered saline solution at a pH of 7.4; such crystals were postulated to be a form of carbonated apatite. They attributed the deposition of these crystals to a nucleation reaction triggered by calcium oxide impurities.

The formation of carbonated apatite could also be achieved by the reaction of calcium carbonate and calcium phosphates under the appropriate conditions [22]. The fact that the apatite-like crystals were not detected when the C− substrates (lacking $CaCO_3$) were exposed to the calcium phosphate solution suggests that the calcium carbonate found in C+ due to the CO_2 treatment played a crucial role in the formation of these crystals. Furthermore, the high concentration of carbon, oxygen, and phosphorous (as confirmed by EDS mapping) on the crystals implies the deposition of a carbonated type of calcium phosphate, which could potentially increase the bioactivity of Portland cement, and have a great impact on the development of load bearing substitutive materials for bone tissue engineering applications [23–25].

No cell growth was observed on C−. This was attributed to the high pH of these samples (≅12.5) caused by the $Ca(OH)_2$ produced during the hydration of the cement. Calcium hydroxide dissociates, releasing $(OH)^-$ groups into the cell culture media, which are known to have a strong cytotoxic effect on the cells [13–15,26,27].

The formation of $CaCO_3$ in the cement by carbonation positively affected the cytocompatibility of this material. This is expected since calcium carbonate is a widely recognized biocompatible material with a pH close to the physiological value, thereby providing a more compatible environment for the cells to grow [13–15,28,29].

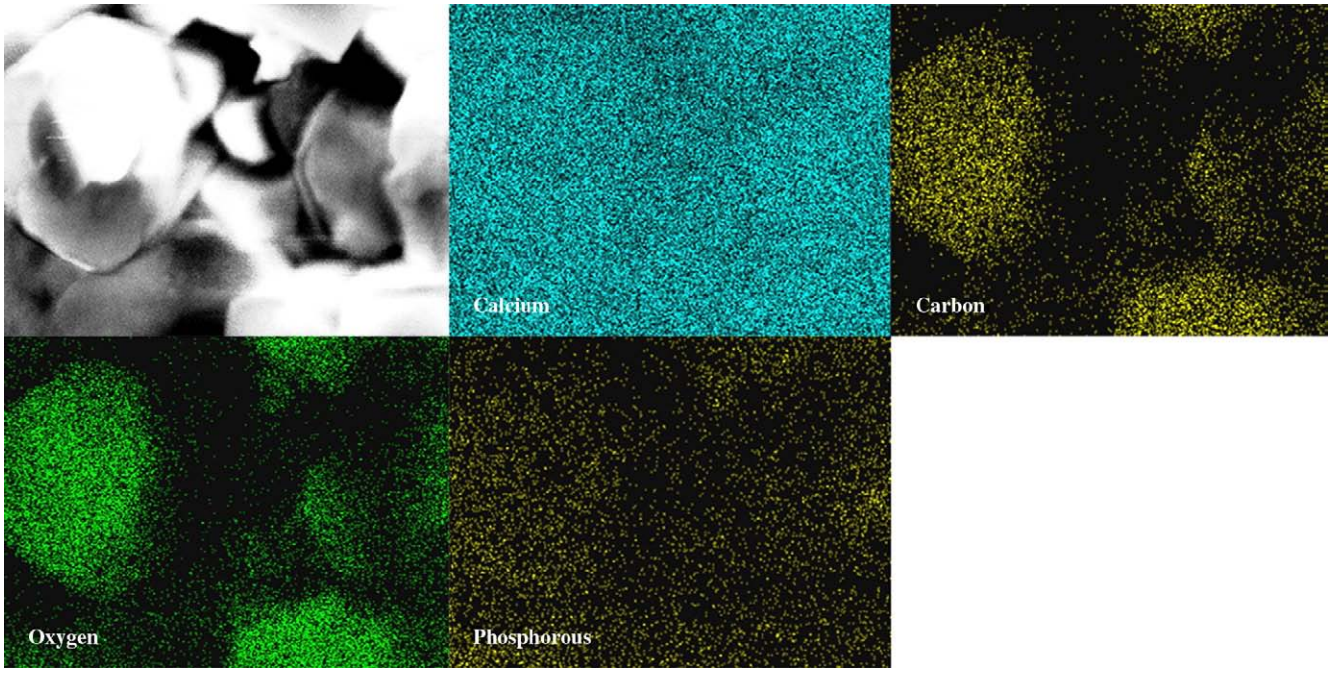

Fig. 6. Distribution of carbon, calcium, oxygen, and phosphorous on the apatite-like crystals precipitated on CPIII.

Cells grew well on the substrates treated with the calcium phosphate solution (CPI, CPII, and CPIII), indicating that these samples had a good degree of cytocompatibility. The cells on CPIII appeared more flattened (compared to C+), which could suggest that these cells presented a higher metabolic and synthetic activity [30], probably due to the presence of calcium phosphates on the sample [31]. However, further research needs to be done in order to verify that this type of substrate induces different cell responses.

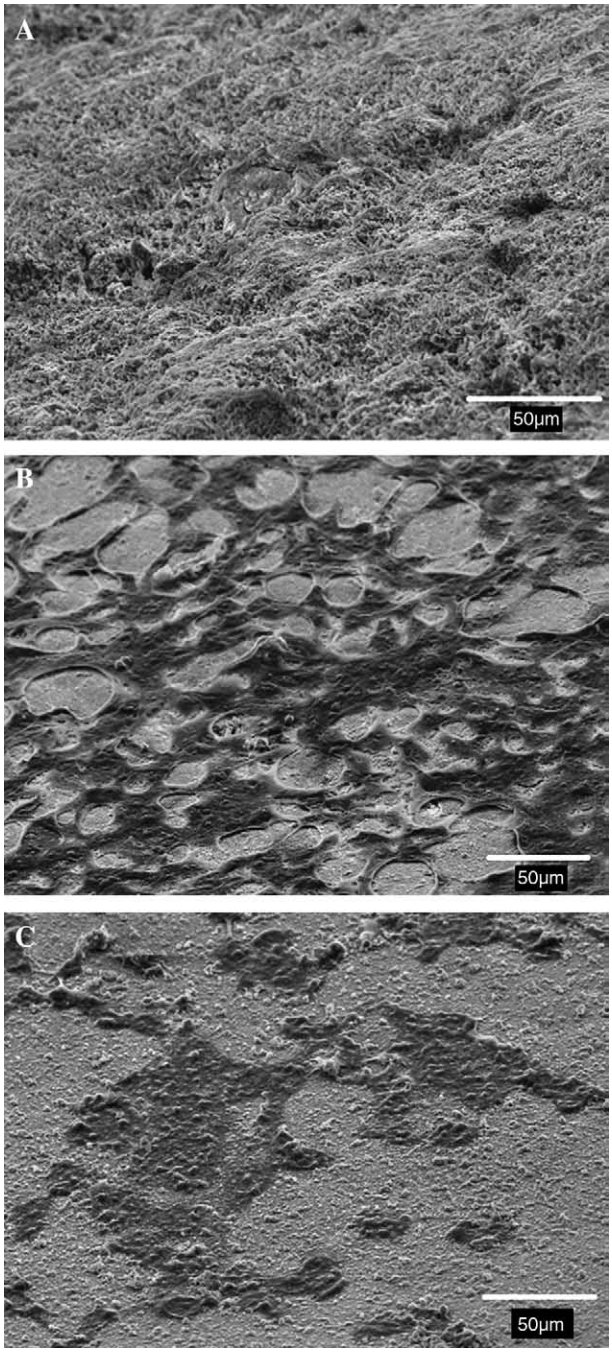

Fig. 7. SEM micrographs of HOS cells cultured on different substrates: A. C−, B. C+, C. CPIII. Images for B and C were selected from areas with lower cell confluence.

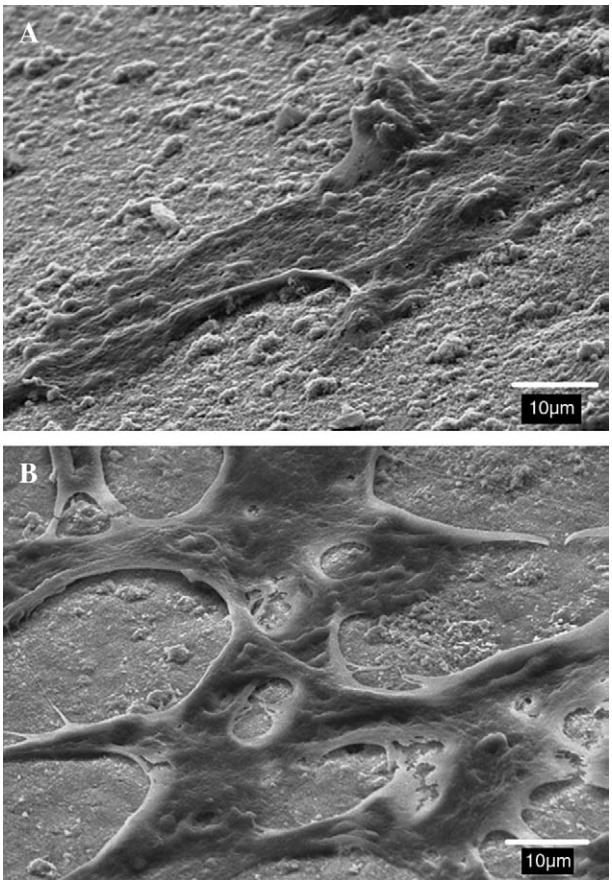

Fig. 8. SEM images of HOS cells cultured on CPIII (A) and C+ (B). It can be observed that the cells on C+ extended more individual cytoplasmic processes, while the ones on CPIII had a more flattened morphology.

5. Conclusion

In conclusion we found that unlike previous reports [32], hydrated Portland cement could be highly cytotoxic due to its basic nature; however, CO_2 treatment counteracted this effect by neutralizing the pH of the material. The exposure of the carbonated cement samples to a calcium phosphate solution promoted the formation of cytocompatible apatite-like crystals on the cement, which could potentially increase the bioactivity of the material, due to the development of bone-like phases.

Acknowledgments

The authors would like to thank Professor Jonas Klemas (posthumous), Nanotech West Laboratory (The Ohio State University), Escuela de Ingenieria de Antioquia (EIA), Instituto de Ciencias de la Salud (CES), Advanced Microscopy Laboratory (UNAL Medellin), Colciencias (Colombia), and Cementos Argos (Colombia).

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