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Behaviour of MG-63 osteoblast-like cells on wood-based biomorphic SiC ceramics coated with bioactive glass

A. de Carlos · J. P. Borrajo · J. Serra · P. González · B. León

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Abstract The aim of this study was to test the *in vitro* cytotoxicity of wood-based biomorphic Silicon Carbide (SiC) ceramics coated with bioactive glass, using MG-63 human osteoblast-like cells, with a view to their application in bone implantology. To better understand the scope of this study, it should be taken into account that biomorphic SiC ceramics have only recently been developed and this innovative product has important properties such as interconnected porosity, high strength and toughness, and easy shaping.

In the solvent extraction test, all the extracts had almost no effect on cellular activity even at 100% concentration, and cells incubated in the bioactive glass-coated SiC ceramics extracts showed a proliferation rate similar to that of the Thermanox control. There were no significant differences when the cellular attachment response of the cells on the wood-based biomorphic SiC ceramics, uncoated or coated with bioactive glass, was compared to the one exhibited by reference materials like Ti6Al4V and bulk bioactive glass. This fact looks very promising for biomedical applications.

1. Introduction

The main challenge of implant technology is to develop materials with enhanced mechanical properties, resistant to wear and with improved physiological response which could be used for load bearing protheses. Today, metallic substrates like titanium and its alloys coated with apatite are common in clinical use [1–5]. In a previous work [6], biomorphic silicon carbide ceramics coated with bioactive glass by Pulsed Laser Deposition (PLD) were proposed as a very promising device for dental and orthopaedic applications. This innovative ceramic substrate is produced by molten-Si infiltration of carbon templates obtained by controlled pyrolysis of wood. Some benefits are expected from its use such as its versatility for the fabrication of complex shapes, its great strength, toughness and intrinsic porosity due to the fibrous nature and microstructure of wood. The biomorphic silicon carbide ceramics retain the micro-structural details of the biostructure-derived carbon preforms and allows the tailoring of a wide range of silicon carbide ceramics with optimised microstructure and properties similar to those of the tissue to be repaired.

Bioactive silica-based glasses are good candidates to be applied as coatings, thereby improving the physiological response of the ceramic substrate because they promote the intimate bonding of living tissues through the formation of a calcium phosphate layer similar to the apatite found in bone [7–9], thus preventing the formation of a fibrous capsule around the implant. Among other coating techniques, i.e. plasma spraying, sputtering, or enamelling; Pulsed Laser Deposition (PLD) has been proven a valid technique to deposit coatings of bioactive materials like hydroxylapatite [10–13] or bioactive glasses [14–20].

According to Kokubo *et al.* [21, 22], the bioactivity of an artificial material can be evaluated by examining the formation, on its surface, of a calcium phosphate layer after immersion in simulated body fluid (SBF), which is a protein-free and acellular solution with ionic concentrations similar to the human plasma. Thus, the bioactivity of silica-based

A. de Carlos
Dpto. Bioquímica, Genética e Inmunología, University of Vigo, 36200 Vigo, Spain

J. P. Borrajo (✉) · J. Serra · P. González · B. León
Dpto. Física Aplicada, University of Vigo, 36200 Vigo, Spain
Tel.: +34-986-812216
e-mail: jpb@uvigo.es

glasses can be assessed by determining the thickness of the silica rich and calcium phosphate layers formed on its surface.

Testing for cytotoxicity is a good first step towards ensuring the biocompatibility of a medical device. A negative result indicates that a material is free of harmful extractables or has a quantity of them insufficient to cause acute effects with isolated cells under exaggerated conditions. However, it is certainly not, on its own merit, evidence that a material can be considered biocompatible - it is simply a first step. On the other hand, a positive cytotoxicity test result can be taken as an early warning sign that a material contains one or more extractable substances that could be of clinical importance. In such cases, further investigation is required to determine the utility of the material.

The main aim of this work is to test the suitability of PLD bioactive glass coated biomorphic SiC ceramics for supporting osteoblast growth and metabolism using the MG-63 cell line model *in vitro* and its comparison with reference materials. The test on extracts was assessed by measuring the cellular activity in response to different concentrations of solvents extracted from the materials using the MTT assay. As a direct contact test, the morphologic characteristics and the attachment rate of MG-63 osteoblast-like cells on different bioactive glass-coated biomorphic SiC ceramics were determined by Scanning Electron Microscopy (SEM) observations. A previous step to *in vitro* studies with MG-63 cells was carried out to select the most bioactive coating obtained by PLD from different glass compositions by means of immersion in SBF.

2. Materials and methods

2.1. Specimen preparation

The biomorphic silicon carbide ceramics were prepared from beech (*Fagus sylvatica*), eucalyptus (*Eucalyptus globulus*) and sapelli (*Entandrophragma cylindricum*). The wood pieces were dried in an oven and pyrolyzed in an alumina furnace in argon flowing atmosphere at 1000°C with well-controlled heating and cooling ramps. The carbonaceous porous preforms were then shaped and infiltrated with pure silicon at 1550°C in vacuum conditions for 30 min. The final plates were cut to obtain pieces of 4.2×4.2 mm^2 and 2 mm in thickness.

Those pieces of each biomorphic SiC ceramics obtained from beech, eucalyptus and sapelli woods, and Ti6Al4V substrates (discs with 5 mm in diameter and 1 mm thickness) were coated with bioactive glass by Pulsed Laser Deposition. Three different glasses in the system SiO_2-Na_2O-K_2O-CaO-MgO-P_2O_5-B_2O_3 (Table 1) were used as targets in a PLD system described elsewhere [16, 17]. The coatings were grown in a high vacuum chamber by irradiation of the glass targets with an ArF excimer laser ($\lambda = 193$ nm). The laser was operated at a repetition rate of 10 Hz providing an energy density of 4.2 Jcm^{-2}. The substrates were kept at a constant temperature of 200°C during the film growth. In order to avoid drilling during the laser ablation, the targets have been rotated at a frequency of 3 rpm.

Table 1 Composition of the glasses (wt%)

Glass	SiO_2	Na_2O	K_2O	CaO	MgO	P_2O_5	B_2O_3
BG42	42	20	10	20	5	3	–
BG50	50	15	15	15	2	–	3
BG55	55	21	9	8	2	4	1
BG59	59	10	5	15	5	3	3

2.2. Bioactivity studies

The bioactivity of the coatings was evaluated by soaking in simulated body fluid. The containers with the samples were kept in an incubator at 36.5 ± 0.5°C.

After 72 hrs of immersion in SBF, the formation and the thickness of the silica-rich and calcium phosphate layers were evaluated and measured by Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectrometry (EDS).

2.3. Biocompatibility studies

2.3.1. Cell culture

The MG-63 human osteoblast-like cell line (ATCC number CRL 1427) was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). The cells were regularly cultured in Earle's modification of Eagle's Medium (EMEM) supplemented with 10% of foetal calf serum, 1% antibiotics, L-glutamine and vitamin C, at 37°C and 5% of CO_2 in a humidified atmosphere.

2.3.2. Solvent extraction test

Pieces of each biomorphic SiC type, and Ti6Al4V substrates, all coated with bioactive glass, were extracted by rolling at 37°C for 90 hrs in EMEM culture medium supplemented with 10% foetal calf serum. A surface area to volume ratio of 3 cm^2/ mL was used. Extracts obtained from Polyvinyl chloride discs (Portex Ltd, UK) and Thermanox plastic cover slips (Nalge Nunc International) were used as positive and negative controls respectively. The extracts were diluted with EMEM to give 10, 20, 30, 50 and 100% of the original concentration. MG-63 osteoblast-like cells were seeded at a concentration of 6×10^5 cells per mL, grown to

confluent layers in 96-well tissue culture plates in a final volume of 0.1 mL of culture medium per well. The different concentrations of the extracts were incubated with cells for 24 hrs. Four wells per substrate and extract concentration were used. After incubation, the cellular activity was quantified by using the MTT assay as previously described [23].

2.3.3. Attachment and proliferation of cells

The samples were placed, under sterile conditions, in 24-well TCP plates. The sterilisation procedure of the discs was performed by dipping the specimens in a 70% ethanol solution and further air drying. This procedure allowed cell growth without detectable contamination signs throughout the experiments. Three replicas from biomorphic SiC ceramics obtained from beech, eucalyptus and sapelli woods coated with bioactive glass, uncoated samples from the same woods, Ti6Al4V and bulk bioactive glass were used to grow MG-63 osteoblast-like cells and were compared at different times after seeding using SEM. After each incubation period (1, 6 and 24 hrs), the pieces were rinsed three times with phosphate-buffered saline (PBS) and fixed with 2,5% glutaraldehyde in 0.1 M cacodylate buffer for 2 hrs. After fixation, the pieces were washed three times with PBS and sequentially dipped in 30, 50, 70, 80 and 95% ethanol for 30 min each and in 100% ethanol for 1 hr. The pieces were submitted to critical point-drying in an increasing ethanol-amylacetate mixture (3:1, 1:1, 1:3, 15 min each) and in pure amylacetate for 15 min, twice, and finally vacuum-dried. A thin layer of gold was sputter-coated onto the pieces prior to examination under a Philips XL30 scanning electron microscope.

3. Results

3.1. Bioactivity studies

A previous bioactivity study of glass coatings grown by PLD from bioactive glasses with different compositions (Table 1) is shown in Fig. 1. After immersion in SBF for 72 hrs, the samples were examined in cross-section by SEM and the thickness of the bioactive layers developed in the physiological fluid was measured.

It can be observed that the higher thickness of the silica-rich and pure calcium phosphate layers is obtained for the coating produced from glass BG42. For glass BG50, a thin pure CaP film appeared on the coating surface followed by a mixed CaP-SiO_2 layer, indicating a lower bioactivity grade. The thickness of the silica-rich layer decreases from BG50 to BG55 and the pure CaP layer was not detected for BG55.

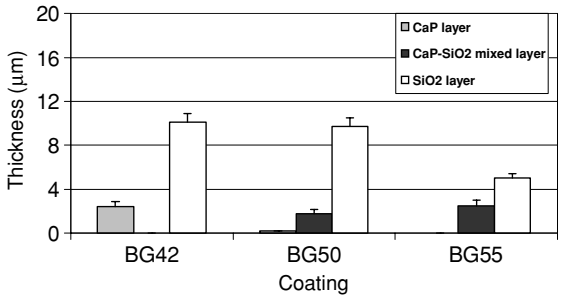

Fig. 1 Thickness of the CaP, CaP-SiO_2, and SiO_2-rich layers originated by the immersion of the bioactive glass coatings in SBF for 72 h.

Finally, for the glass BG59 coating (not shown), no bioactive products were observed. Thus, it can be concluded that the PLD bioactive glass coating with an optimal grade of bioactivity should be grown from glass BG42.

This behaviour is due to the open amorphous structure of the silica-based glasses which enables the accommodation of alkali and alkali-earth cations. These modifiers provoke the disruption of the continuity of the glassy network due to the creation of local defects leading to the formation of non-bridging oxygen groups (Si-O-NBO's). It has been demonstrated [24, 25] that the concentration of NBO's groups is a key factor that determines the bioactivity grade of the silica-based glasses because it controls the dissolution rate of the glass, when immersed in physiological fluids, through the formation of silanol groups at the glass surface. The most bioactive coatings, showing the thickest CaP and silica-rich layers, were grown from glass BG42 which exhibits the higher concentration of network modifiers and, consequently, higher amount of NBO's groups.

3.2. Biocompatibility studies

3.2.1. Solvent extraction test

To assess the cytotoxicity of this innovative product, beech, eucalyptus and sapelli-based biomorphic SiC ceramics have been coated by PLD with the previously selected bioactive glass BG42.

Fig. 2 summarises the MG-63 osteoblast-like cell activity after incubation with different concentrations of the extracts obtained from coated biomorphic SiC ceramics, Thermanox plastic cover slips used as negative control and PVC used as positive toxic control. The mitochondrial enzyme succinate dehydrogenase, only present in viable cells, converts the MTT molecules into purple coloured formazan crystals. The amount of colour is measured by means of its corrected absorbance value at 570 nm (A_{570}–A_{700}) and is proportional to the number of viable cells. The mean corrected absorbance values obtained are represented. The

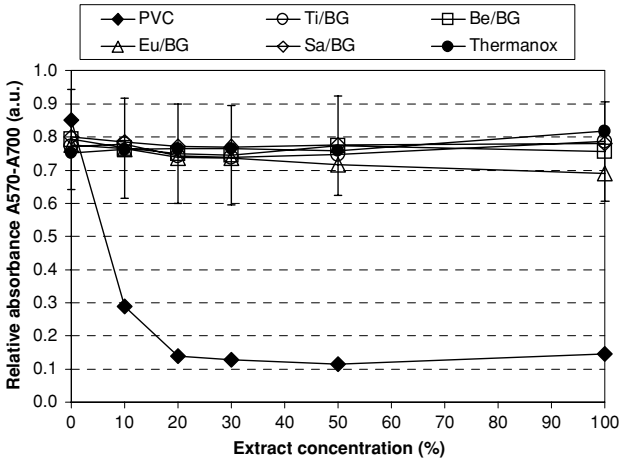

Fig. 2 Relative cellular activity from the solvent extraction test. Thermanox: negative control; PVC: positive toxic control; Ti/BG: Ti6Al4V substrate coated with bioactive glass; Be/BG, Eu/BG, Sa/BG: beech, eucalyptus and sapelli-based SiC ceramics coated with bioactive glass, respectively.

representation shows that the PVC extract was cytotoxic for the MG-63 monolayer at all the concentrations tested, thus validating the extraction procedure. The bioactive glass coating extract did not affect cellular activity at any of the concentrations tested, their values being similar to the ones obtained for the Thermanox cytotoxic negative control. A slight decrease in cellular activity could be observed for the 100% concentration of the bioactive glass-coated eucalyptus SiC ceramic extract, which was not over the significant limits. In the case of the extracts obtained from the other two wood-based biomorphic bioactive glass-coated SiC ceramics (beech and sapelli), no detrimental effect could be seen at any of the concentrations tested.

3.2.2. Time course of monolayer formation

Fig. 3 offers, at a 1000x magnification, scanning electron microscopy images showing the MG-63 osteoblast-like cell monolayer time course formation. A, 1 hr; B, 6 hrs and C, 24 hrs after seeding on a representative beech-based SiC ceramic coated with bioactive glass. One hour after seeding (A), rounded cells involved in cellular division events can be seen attached to the outer surface inside the pores. Cells begin to penetrate and colonise the inner surface of the existing pores. At 6 hrs after seeding (B), cells are attached and have spread out, displaying a flat configuration and a normal morphology. Neighbouring cells maintained physical contact with one another through extensions of the cytoplasm. At 24 hrs (C), the bioactive glass coated surface was almost completely covered by the MG-63 cells. No evidence of major deleterious or cytotoxic responses was observed. The biomorphic beech-based SiC ceramics coated with bioactive glass supports the cellular monolayer formation and the colonisation of the surface of the material. The same results were obtained for the eucalyptus and the sapelli-based coated ceramics.

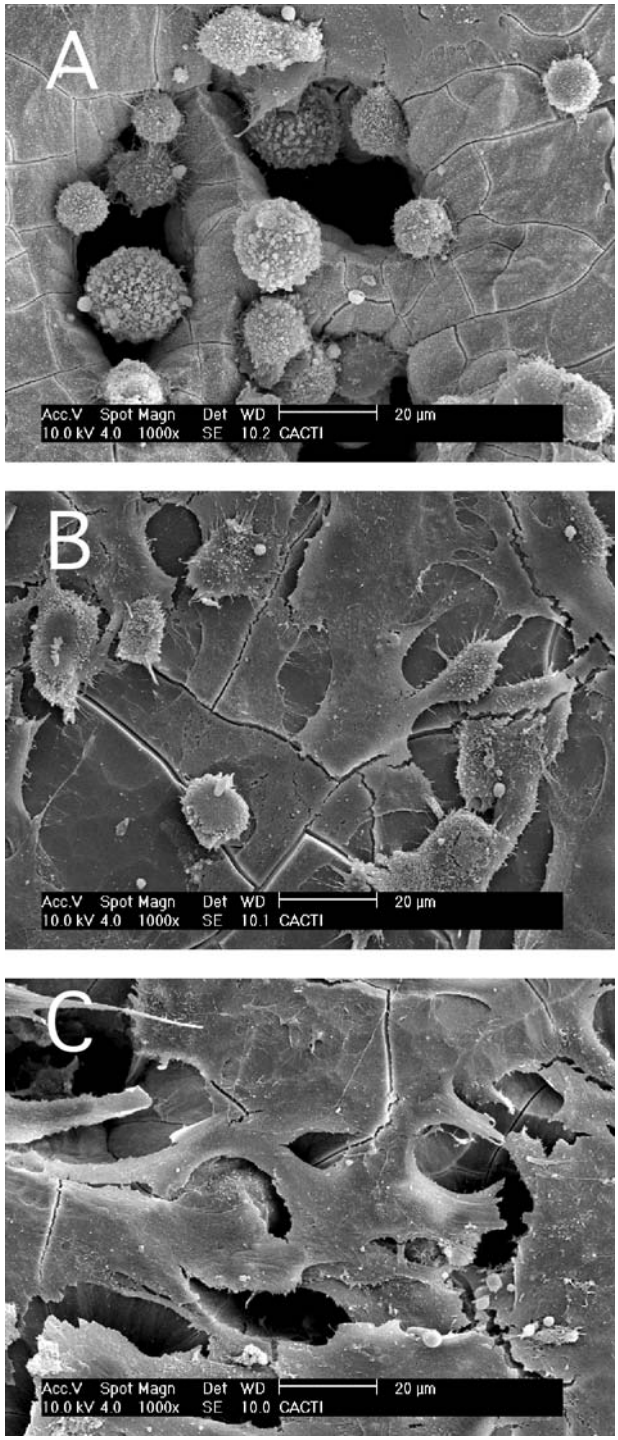

Fig. 3 Scanning electron microscopy images showing the MG-63 osteoblast-like cell monolayer time course formation. A, 1 hour; B, 6 hrs and C, 24 hrs after seeding on a representative beech-based SiC ceramic coated with bioactive glass. All magnifications are 1000×.

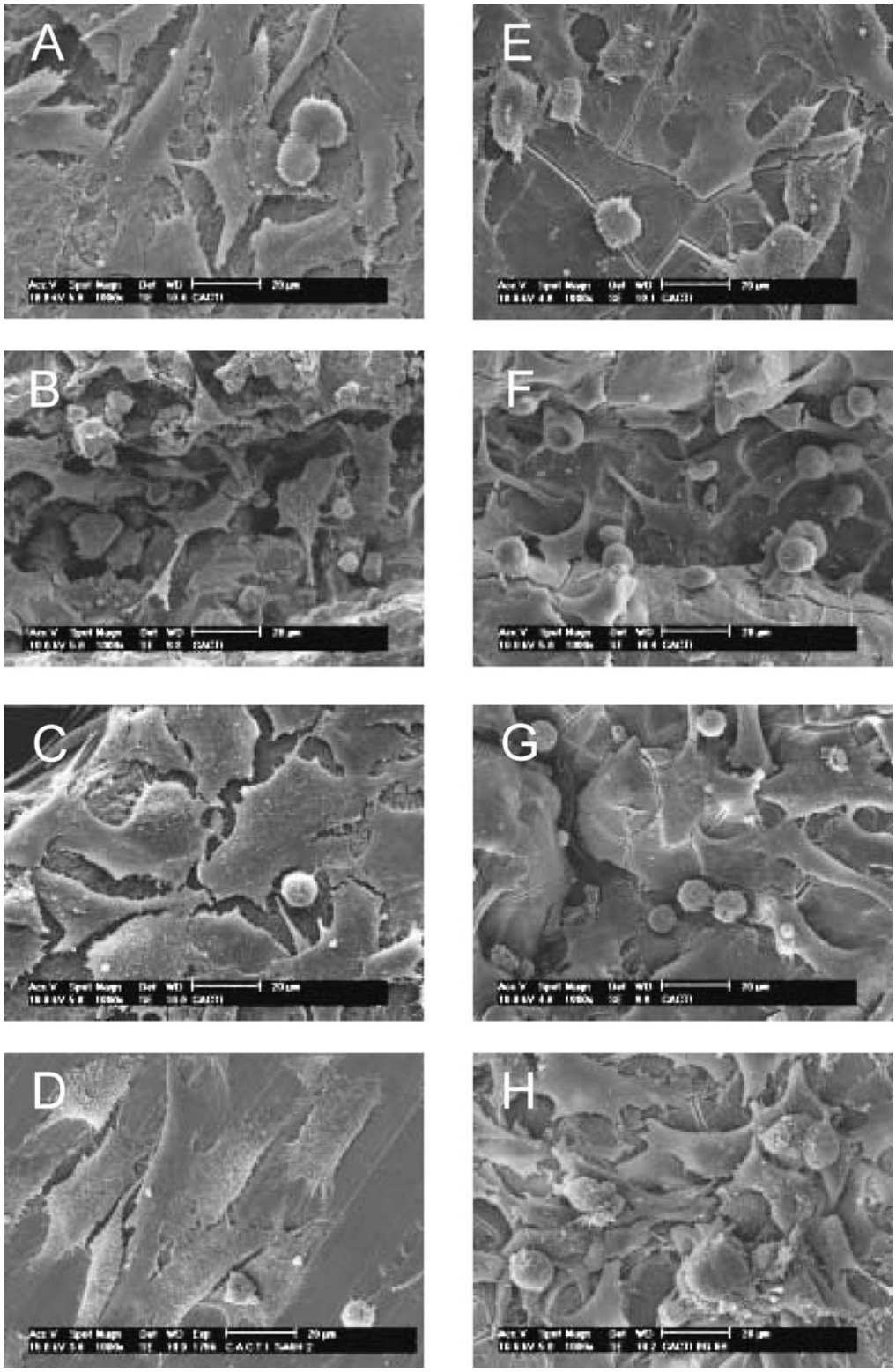

Fig. 4 Scanning electron microscopy images showing the comparative attachment of MG-63 osteoblast-like cells 6 hrs after seeding on uncoated (A, B, C) or bioactive glass-coated (E, F, G) beech (A, E), eucalyptus (B, F) and sapelli (C, G) - based biomorphic SiC ceramics, and on two reference materials, Ti6Al4V (D) and bioactive glass (H). Magnifications in all cases are 1000×.

3.2.3. Cell attachment and material surface comparisons

The attachment of MG-63 osteoblast-like cells on beech, eucalyptus and sapelli-based biomorphic SiC ceramics coated with bioactive glass was compared by means of SEM, 6 hrs after seeding and the results are shown in Fig. 4. Uncoated samples were examined for comparison as well. Provided that the same number of cells have been seeded, the attachment of the cells occurs in the same way in all tested samples. None of the wood-based SiC ceramics coated or uncoated exhibited a higher rate of cell attachment and growth. The cells attached in the same efficient manner to all parts of the ceramic pieces, including naturally occurring pores and channels present on axial (A and C, upper right angles) and longitudinal (B, F) sections of uncoated (A, B, C) or coated (E, F, G) samples. At this magnification (1000x), where cell details become more conspicuous, flattened and spread osteoblast-like cells were observed. Expansion of the cytoplasm was already visible and completely spread with, in many cases, the bulge of the nucleus and surface microvilli very apparent, with profusion of filopodia, as well as larger cytoplasm extensions (lamellipodia). Neighbouring cells maintained physical contact with one another through cytoplasm extensions. No evidence of any major deleterious or cytotoxic responses was observed. The appearance of the MG-63 cells was the same as the one observed on two reference materials like Ti6Al4V (D) and bulk bioactive glass (H).

4. Discussion

The main objective of this study was to test the *in vitro* cytotoxicity of biomorphic SiC ceramics coated with bioactive glass towards their application in bone implantology by determining the biological response of the MG-63 human osteoblast-like cell line. With its osteoblast-like phenotype, the MG-63 cell line has served as a useful model to test the biological performance of various materials [23, 26], has allowed the study of cell-surface interactions [27] and furthermore has proved its ability to proliferate on bioactive glass [28, 29].

The behaviour of foetal rat osteoblast cultured upon bioactive glass cultures has shown compact cells with dorsal ruffles and filopodia resulting in the formation of a denser cell layer [30], better osteoblast-like morphology and a higher proliferation rate, leading to confluent cultures with higher cell density and a generally better expression of the osteoblast phenotype in comparison with substrates like hydroxylapatite, a titanium alloy and stainless steel [31].

Bioactive glass also promoted neonate rat calvaria to colonize samples in multilayers and produce abundant extracellular matrix developing collagen and non-collagen bonding with the rich calcium phosphate-rich glass [32], representing a material that optimally combines the requirements of the ideal template for in vitro synthesis of bone tissue [33].

The biocompatibility of bioactive glass coatings as well as their osteoconductive properties have been also assessed by employing primary cultures of human osteoblasts, resulting effective in stimulating osteoblast growth and differentiation [34]. Using cDNA microarray analysis, the gene-expression profiling of human osteoblasts, following treatment with the ionic products of bioactive glass dissolution, has been investigated, resulting in an increase of the expression level of many different genes, markedly cell cycle regulators such as cyclin D1 and apoptosis regulators, including calpain and DAD1 as well as cell surface receptors like CD44 and integrin $\beta 1$ and various extracellular matrix regulators, including metalloproteinases and other osteoproductive-related genes [35].

There are few studies using human osteoblast-like cells MG-63, but those existing support the hypothesis that bioactive glass provides a favourable environment for human osteoblast proliferation and function [28].

In this investigation, none of the bioactive glass coated SiC ceramics extracts showed significant cytotoxic reaction using the MTT test to compare the cellular activity of the MG-63 cells when challenged even at the highest concentration.

Time course experiments were performed to verify cellular behaviour with respect to cell attachment and growth on these coated surfaces. All the wood-based SiC ceramics coated with bioactive glass supported the growth of the osteoblast-like cells in a 24 hrs time course in the same way.

Comparison of the cellular attachment and morphology 6 hrs after seeding on the different uncoated or coated ceramics with two reference materials like Ti6Al4V and bulk bioactive glass led to the conclusion that all the materials behaved in the same non cytotoxic way and exhibited attachment and growth rates similar to those of the reference materials.

5. Conclusions

The biocompatibility of this innovative product based on biomorphic SiC ceramics has been demonstrated. SiC ceramics coated with bioactive glass showed the same biological response as the reference materials Ti6Al4V and bulk bioactive glass. The biomorphic SiC ceramics coated with bioactive glass by PLD did not produce a cytotoxic response on the MG-63 osteoblast-like cells. The same behaviour was observed for uncoated ceramics. The cellular activity on coated and uncoated SiC ceramics was similar to well known implant materials like Ti6Al4V and bulk bioactive glass.

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