Osteoblastic cell response to SPS-sintered zirconia/titanium cermets

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***Published in Journal of Biomaterials Applications 2015, Vol. 29(6) 813–823**

Abstract

Ceramic/metal composites, cermets, arise from the idea to combine the dissimilar properties in the pure materials. This work aims to study the biocompatibility of new micro-nanostructured 3Y-TZP/Ti materials with 25, 50 and 75 vol. % Ti, which have been successfully obtained by Spark Plasma Sintering (SPS) technology; as well as to correlate their surface properties (roughness, wettability and chemical composition) with the osteoblastic cell response. All samples had isotropic and slightly waved microstructure, with sub-micrometric average roughness. Composites with 75 vol. % Ti had the highest surface hydrophilicity. Surface chemical composition fitted to the nominal amounts used for the fabrication. A cell viability rate over 80% dismissed any cytotoxicity risk due to manufacturing. Cell adhesion and early-differentiation were significantly enhanced on materials containing the nanostructured 3Y-TZP phase. Proliferation and differentiation of SaOS-2 were significantly improved in their late-stage on the composite with 75 vol. % Ti that, from the osseointegration standpoint, is presented as an excellent biomaterial for bone replacement. Thus, SPS is consolidated as a suitable technology for manufacturing nanostructured biomaterials with enhanced bioactivity.

Keywords

Zirconia, titanium, nanostructured, spark plasma sintering (SPS), biocompatibility.

Introduction

Since the development of the first devices used for bone replacement in 1950¹, endosseous prostheses, like dental implants or articular prostheses have been fabricated from a wide variety of biomaterials, essentially of ceramic or metallic nature.^{2, 3} Traditionally, metal devices have been used as bone replacement materials mainly because of their superior mechanical properties, especially high toughness and strength.³ Among them, titanium (Ti) is the most used metallic material due to its high biocompatibility and osseointegration capacity.^{4, 5} On the other hand, oxide ceramics such as alumina and zirconia have been extensively used in prosthetic devices due to their high wear resistance and outstanding compressive strength and hardness.^{6, 7} Among them, zirconia stands out for its high fracture toughness resulting from the transformation of metastable tetragonal grains to the monoclinic phase in the presence of stress induced by the evolution of a crack tip.^{8, 9}

Both ceramics and metals present multiple advantages but also significant drawbacks,^{1, 2} which make it necessary to develop new materials that meet certain properties that allow to solve some of the problems related to the biomechanical incompatibility with bone.¹⁰ Composites are a well-known way of achieving multiple improved features with respect to the individual components.¹¹ Ceramic/metal composites (cermets) have been developed to combine favourably the dissimilar properties of ceramic and metal counterparts.¹²⁻¹⁶ Cermets can be presented as a new generation of biomaterials for hard tissue replacement because they possess a combination of functionalities including electro-machinability, fracture toughness, wear resistance, hardness and damage tolerance.¹²⁻¹⁵ Thus, the application of this type of composites for bone replacements opens the possibility of improving several mechanical features to a greater extent than using the single components.

In this case, zirconia and titanium were the components selected because of their good integrative behaviour within host tissues that makes them two suited and widely used biomaterials in dental and orthopaedic fields.^{2, 6, 7, 17-21} These materials had never been combined in a composite so far, because during classical sintering procedures both phases react each other and with the gas in the atmosphere, forming side compounds that weaken the structure.^{22, 23} However, by using SPS as alternative sintering technology, a 3Y-TZP/Ti system has been recently developed.²⁴ Spark Plasma Sintering (SPS) has evolved as a consolidation method for new materials that are difficult to manufacture by conventional techniques. SPS technology allows to apply electrical pulses and uniaxial pressure simultaneously within vacuum conditions; this along with the high heating rates (100 °C/min) facilitates a fast material consolidation, reducing reactivity and maintaining a grain size similar to the starting powders. Previous studies have demonstrated that composites including nano-sized particles reduce the production of wear debris, resist crack propagation²⁵ and have a better biological response.²⁶ In this line, a Ti micrometric powder and a 3Y-TZP nanoparticle aggregate powder were used as raw materials in order to produce SPSed micro-nanostructured composites. The ability to successfully develop these novel 3Y-TZP/Ti composites has encouraged the evaluation of their cytocompatibility. Although there are many reports about *in vitro* and *in vivo* biocompatibility of pure zirconia and titanium,^{4, 20, 27-30} the biological response to 3Y-TZP/Ti cermets has never been studied; the new formulation and also the innovative manufacturing process may lead to new unexpected biological responses that must be assessed.

The relative amount of Ti and 3Y-TZP varied in the composites allowing to study the effect of chemical composition and manufacturing process on surface properties and cell behaviour. It is well-known that surface properties include specific physico-chemical aspects with a crucial role on the material-tissue interactions; roughness, chemical composition and wettability are among

the main inherent factors to surfaces that influence the protein adsorption, the subsequent cell attachment and, therefore, the final material osseointegration.^{28, 31, 32} The first cell-surface interactions define the quality of the tissue-device interface because they lead transduction signals to the nucleus that will induce cell adhesion, followed by proliferation³³ and differentiation³⁴, or apoptosis³⁵ when adhesion is not facilitated. Although *in vitro* assays can never substitute *in vivo* experiments, they provide relevant information on the biological performance of newly developed materials using defined environments for cell-material interactions. Thereby, this study focuses on the analysis of *in vitro* biocompatibility of the novel generation of micro-nanostructured 3Y-TZP/Ti cermets, paying special attention to the effect of their surface properties (topography, wettability and chemical composition) on adhesion, proliferation and differentiation of SaOS-2 osteoblast-like cells. Both 3Y-TZP and Ti counterparts were chosen as reference materials in order to better understand the role played by the individual constituents of the cermet. Further information is obtained about *in vitro* activity of nanostructured zirconia, less known than conventional (micro-structured) ceramics.

Materials

The raw materials were (i) spray-dried zirconia powder (TZ-3Y-E; Tosoh Corp., Japan) with 0.04 μm of particle size, $16 \pm 3 \text{ m}^2/\text{g}$ of specific surface area and very low amount of impurities (wt. %): $\text{HfO}_2 < 5.0$, $\text{Al}_2\text{O}_3 = 0.1\text{-}0.4$, $\text{SiO}_2 \leq 0.02$, $\text{Fe}_2\text{O}_3 \leq 0.01$ and $\text{Na}_2\text{O} \leq 0.04$; and (ii) Grade I commercially pure titanium powder (Ti; Goodfellow, UK) with maximum particle size of 150 μm and 99.5% of purity ($\text{C} < 100 \text{ ppm}$, Chlorides = 1700 ppm, $\text{Fe} = 200 \text{ ppm}$, $\text{N} < 100 \text{ ppm}$, $\text{O} = 1000 \text{ ppm}$). 3Y-TZP/Ti composites (Z-Ti) with three different titanium contents (25, 50 and 75 vol.% Ti) as well as pure 3Y-TZP and Ti were developed following instructions in ²⁴. Table 1 shows the sample code for the diverse materials involved in this study. Briefly, a wet-processing route with an organic deflocculant was used to form stable suspensions that were dried and sieved getting

well-dispersed powders. The resulting powders were sintered within vacuum at 1350 °C, 80 MPa, 10 min with a heating rate of 100 °C/min by Spark Plasma Sintering (SPS) in order to conform dense specimens.²⁴ The remnants of the graphite foil used in the sintering process were completely removed from the SPSed blanks with an 80 grit SiC grinding cloth. Then, samples were machined to 11.6x11.6x2 mm plates and subsequently polished down to 1200 grit. The samples were ultrasonically cleaned up for 15 min in acetone, ethanol and demineralized water and dried at room temperature for 24 h before experiments.

Methods

Surface analysis

Topography/roughness. Since a similar polishing procedure may lead to different topographic characteristics as a result of the diverse nature of the materials, all studied substrates were quantitatively evaluated by scanning electron microscopy (SEM, Hitachi analytical TableTop microscope TM3000; Hitachi High Technologies, Europe). In addition, the ZrO₂ microstructure was assessed by field emission scanning microscopy (FE-SEM with Quanta FEG 650 with S/TEM detector; FEI Company, The Netherlands) on 1µm-polished samples after a thermal treatment (1000 °C, 1 h) in air-atmosphere and Ir-coating in order to confirm the presence of a nanostructured ceramic-phase in the materials containing 3Y-TZP. Furthermore, the three-dimensional micro-roughness pattern was examined in each system by white light interferometry (Veeco Wyko 9300 NT, white light interferometer microscope). Four measurements were randomly taken in each one of the three different samples of each group. The analysis area was 165.2x220.2 µm². Data analysis and filtering were performed with Wyko Vision 4.10TM software (Veeco Instruments, NY, US). A Gaussian filter was used to eliminate waviness and tilt. Cut-off (λc) values of 0.08 mm were applied. The values of all individual profiles evenly distributed along the surface of analysis were averaged to reconstruct the 3-D

images of the surfaces and calculate roughness parameters. The average roughness (R_a) and the mean roughness depth (R_z) were quantified to evaluate potential roughness differences among studied materials. Index area (S_{AI}) and real surface area (RSA) were characterized in order to standardize the cell number in the proliferation tests regarding the real area in the substrate. S_{AI} , the real surface area/nominal surface area, indicates the increase in the real area compared to the nominal area of a sample due to roughness (the higher roughness, the higher index area).^{36, 37}

Wettability. The surface energy in the surfaces affects the biological response to material. This is measured indirectly by the liquid-solid contact angles. A video contact angle system OCA15+ (Dataphysics, Germany) was used to measure the apparent static contact angles (CA) of ultrapure distilled (DI) water on the diverse samples, with the sessile drop method through Young's equation.³⁶ Four measurements were performed, at room temperature, in each of three specimen of each group.

Surface chemical composition. A chemical analysis was conducted by X-ray photoelectron spectroscopy (XPS) with a spectrometer equipped with a Hemispherical Energy Analyzer PHOIBOS 150 (SPECS Surface Nano Analysis GmbH; Berlin, Germany) and Mg X-ray radiation source at 1253.6 eV and scan system control in order to demonstrate the different effects of material elementary composition on cell behaviour. The pressure was 10^{-10} mBar before the data were recorded and the current of the anode was 10 kV. The electron gun focused on an area close to 1 mm. Four survey-scans were recorded per sample (0-1100 eV) at 1eV step-size. Curve fitting and elemental (atom %) semi-quantification from the surveys were performed with the software Casa XPS, V 2.3.16 (Casa Software Ltd., 2005).

In vitro biocompatibility assays

Cell culture. All reagents were obtained from Invitrogen (Carlsbad, CA, US). The human osteoblast-like SaOS-2 cells (ATCC, Manassas, VA, US) were cultured in McCoy's 5A supplemented with 15% fetal bovine serum (FBS), L-glutamine (1.5 mM), sodium pyruvate (2 mM) and penicillin/streptomycin (50 U/ml and 50 µg/ml, respectively) at 37°C in a humidified incubator at 5% CO₂, changing the medium every 2 days. Subconfluent cells were trypsinized using a 0.05 % trypsin-EDTA solution and centrifuged.

Cytotoxicity test: Water-soluble Tetrazolium Salt (WST-1). Cytotoxicity tests were based on ISO 10993-5.³⁸ Briefly, three samples of each group were incubated in supplemented phenol red-free McCoy's 5A culture medium (Promocell; Labclinics SA, Spain) for 72 h at 37°C. SaOS-2 cells (5×10^3) were grown for 24 h and then incubated in presence of the complete extracts (named as extraction vol.) and their diluted extracts (ranging from 1:1 to 1:1000). After different exposure periods (24 and 72 h), the colorimetric assay WST-1 (*Cell Proliferation Reagent*; Roche Applied Science, Penzberg, Germany) was used for cell quantification. The reagent WST-1 (diluted 1:10 in serum free McCoy's 5A without phenol red) was added to the cells for 2 h. Mitochondrial dehydrogenases in metabolically active cells cleaved this slightly yellow tetrazolium salt, (4-[3-(4-Iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate), to dark yellow formazan that was assessed at 440 nm using the PowerWaveX spectrophotometer with the Gen5 Data Analysis software (Bio-Tek Instruments, Inc.; Winooski, VT, US).

Cell adhesion: morphology of adherent cells and presence of focal adhesions. Cells (20×10^3) were seeded onto the samples, which had been previously sterilized within ethanol (70%, v/v), rinsed with PBS and incubated in FBS for 24 h. Agarose-coated 24-well plates were used, to prevent cell attachment on the well. Cell spreading at 24 and 48 h was studied by the *Actin Cytoskeleton and Focal Adhesion Staining Kit*, FAK 100 (Millipore; Billerica, MA, US), following manufacturer's instructions. Briefly, cells were fixed for 20 min in 4% PFA and permeabilized for 15 min in 0.1%

Triton X-100. Cells were blocked using 1% BSA (in 1xDPBS) for 1 h and incubated for 2 h with 1:200 mouse anti-vinculin. Alexa Fluor® 488 Goat anti-mouse IgG (1:1000; Invitrogen, Life Technologies Corp.) and TRITC-conjugated Phalloidin (1:300) were simultaneously applied for 1 h in dark. Nuclei were stained using DAPI (1:1000) for 2 min. At least five images were randomly acquired from each assay condition by an E600 fluorescence microscope (Nikon, Japan) at 20x and treated with Cell F software (Olympus, Japan). Micrographs were examined using ImageJ software (<http://rsbweb.nih.gov/ij/>) to calculate cell-shape parameters.^{10, 39} Specifically, cell area was quantified to study cellular spreading and circularity to assess cellular morphology. Circularity was calculated according to Equation (1) so that, if values tend to one, cell shape is more circular than if they tend to zero.

$$\text{Circularity} = \left[\left(\frac{\text{area}}{\text{perimeter}^2} \right) \times 4\pi \right] \quad (1)$$

In addition, vinculin staining was visualized using a confocal laser scanning microscopy TCS-SP5 (Leica Microsystems; Wetzlar, Germany).

Cell proliferation. Cells (10×10^3) were seeded onto each sample, previously sterilized and pre-incubated in FBS. After 1, 3, 7 and 14 day/s, the samples were rinsed with PBS and the cells were lysed with 500 µl M-PER® (Mammalian Protein Extraction Reagent; Thermo Scientific, Waltham, MA, US). Cell proliferation was evaluated by measuring the lactate dehydrogenase (LDH) cytoplasmic activity with the *Cytotoxicity Detection Kit^{PLUS}* LDH (Roche Applied Science; Penzberg, Germany). The reduction of tetrazolium salts into formazan dye, a reaction coupled to LDH activity, was measured spectrophotometrically (492 nm). An increase in the absorbance directly correlates with an increase in cell number. The number of cells at each culture time was obtained from the extrapolated OD₄₉₂ values in a calibration curve of increasing numbers of cells (ranging from 0 to 50×10^3 cells). Results from each surface were normalized with the corresponding S_AI index calculated from interferometry.

Osteogenic differentiation: Alkaline phosphatase activity (ALP). The osteoblastic differentiation of SaOS-2 cells cultured on the surface of the Z-Ti composites or the individual counterparts was evaluated by quantification of alkaline phosphatase (ALP) activity by means of the *Sensolyte pNPP Alkaline Phosphatase Assay Kit* (AnaSpec Inc.; Fremont, CA, US). Briefly, cells (10×10^3) were seeded onto the different materials. After 24 h, the medium was supplemented with 50 µg/ml of ascorbic acid, 10 mM β-glycerophosphate and 100 mM dexamethasone (Sigma-Aldrich; St. Louis, MO, US). Cells were lysed after 1, 3, 7 and 14 day/s with 500 µl M-PER® (Thermo Scientific, Thermo Fisher Scientific, Inc.; Waltham, MA, US) and cellular extracts were incubated for 15 min at room temperature with the colorimetric ALP substrate pNPP. The absorbance was measured at 405 nm and DO values were extrapolated to a calibration curve of ALP standard from calf intestine provided by the kit (serial dilutions from 1000 to 0 ng/ml). Results were normalized with the cell number obtained from LDH activity and the corresponding incubation period.

Statistical analysis

Data are presented as the mean value with the corresponding error of three independent experiments, except where otherwise stated, to ensure consistency. Significant differences of *in vitro* assays were analyzed by ANOVA tests followed by *post hoc* pairwise comparisons using Tukey-HSD or Dunnett-C depending on the homogeneity of the variance. Differences were also analyzed using Kruskal-Wallis non-parametric test followed by Mann-Whitney test with Bonferroni correction with very similar results. Statistical tests were performed with SPSS statistics v20 (IBM, Armonk, NY, US).

Results and discussion

Surface characterization

Topographical analyses. SEM analysis showed two well-dispersed and clearly distinguished phases in the 3Y-TZP/Ti composites. These substrates presented an overall smooth topography interspersed with irregular areas of valleys associated preferentially to titanium particles; the softest phase in the composite. Z-0Ti and Z-100Ti pure counterparts showed longitudinal parallel grooves resulting from the polishing, which were also visible in Z-75Ti cermets, although less marked. Figure 1 shows a representative FE-SEM micrograph of Z-0Ti substrates, disclosing the characteristic nanosized grains of SPSed monolithic 3Y-TZP.

Figure 2 presents the interferometric 3-D images, as well as the mean and SD values of the evaluated roughness parameters. Although all samples had an isotropic 3D-topography, with a similar wavy pattern in all directions, differences in roughness values were found between substrates at the sub-micrometric level. Several studies have demonstrated that osteoblast adhesion is influenced by surface roughness, particularly when the roughness ranges in the submicron to the micron scale.⁴⁰ Z-25Ti and Z-75Ti specimens presented lower R_a values than the rest of the substrates. Higher R_a values matched with higher RSA and S_{Al} values, as expected from rougher surfaces. Higher R_z values were found for Z-50Ti and Z-100Ti surfaces.

Wettability. Previous works have reported that, in overall, hydrophilic surfaces in contact with blood and biological fluids promote protein adsorption in a conformation that exposes adhesion motifs and enhances cell behavior^{10, 41}. Thereby, osteoblasts would adhere and proliferate better on hydrophilic substrates⁴², although the optimal degree of hydrophilicity remains unclear.^{41, 43} Table 2 summarizes the mean and SD values of CA of DI-water on the different materials. The most hydrophilic surface was Z-75Ti, with a CA of approximately 54°. The rest of the materials exhibited less hydrophilic behaviour, with CA values higher than 85°. The solid-liquid interactions are affected by the surface type (porosity, roughness and crystallinity). In particular, such interactions strongly depend on the chemical composition that determines polar (dipole-dipole and hydrogen bonding) and electrostatic (ion-dipole) interactions⁴⁴. Z-75Ti specimens were the cermets that

presented the highest reactivity in the sintering process ²⁴; therefore it is not strange to find different wettability properties in these substrates. ⁴⁵

Chemical composition of the surfaces. XPS analyses (Figure 4) did not show any additional element on the surfaces to those derived from the chemical composition of the bulk material: Ti, Zr, Y, O; except C from atmospheric contamination. The quantities expressed as % atom are presented in Table 3. The surface chemical composition of the cermets correlated well with the relative amounts used for their fabrication and the amount of Ti (% atom) on Z-Ti surfaces decreased with increasing 3Y-TZP content in the bulk material. A higher O (% atom) content on Z-100Ti samples is related to titanium oxidation. An excess in C (% atom) would be associated with the graphite sheet used in the sintering process.

In vitro biocompatibility assessment

Cytotoxicity test. All new materials intended to be used in medical devices must be assessed in terms of *in vitro* cytotoxicity as per ISO 10993-5:2009.³⁸ Figure 4 presents the cell viability (%) rates, which were higher than 80% on all the cermets, as well as on the 3Y-TZP and Ti monolithic samples. Considering cytotoxicity rates as inversely proportional to cell viability rates, cytotoxicity (%) ratio was below 20% on all the materials when cells grew for 24 and 72 h, either within the material extracts or their dilutions. Hence, a potential cytotoxic effect related to these systems is dismissed.

Cell adhesion behaviour. Figure 5(a) shows the arrangement of the red-fluorescent F-actin network of attached SaOS-2 cells at the studied surfaces, 24 and 48 h after seeding. Osteoblasts presented spindle-shaped morphology and more complex morphologies at the longest culture time (48 h). They also showed a tendency to establish intercellular connections through filopodia. 3Y-TZP and Z-Ti cermets promoted a more elongated shape of osteoblasts than Z-100Ti. Figure 5(b) shows the green-fluorescence of immunolabeled-vinculin disclosing large areas of focal adhesions distributed along the cell borderline 48 h after seeding. Particularly, Z-

Ti surfaces enhanced the expression of this green-immunolabeled protein involved in cellular anchoring.

The actin cytoskeleton also allowed to study the cell spreading on the different materials over the short-term adhesion stage. Figure 6 summarizes mean and SD values of cell area and circularity in the osteoblasts adhered at the different surfaces. At both incubation times, the cell area on the surfaces increased as the 3Y-TZP volume increased in the bulk material, with the area values significantly enhanced on the materials containing 3Y-TZP if compared to Z-100Ti specimens (Figure 6(a)). Regarding morphology, osteoblasts presented a 0.5 circularity value on every surface 24 h after seeding. Cell circularity decreased on all tested substrates at 48 h, to a higher extent in the samples containing 3Y-TZP (Figure 6(b)).

Previous studies^{27, 29} have also demonstrated better osteoblast behaviour on zirconia than on titanium surface. The primary interaction zone with the biological tissues is the outermost atomic layer of a physical device, so the interactions between cells and surfaces will be influenced by varying the surface chemical composition.⁴⁶ Our results suggest that cell spreading on Z-Ti cermets was more influenced by surface chemical composition than by roughness and CA values. All substrates, except Z-75Ti specimens, presented similar CA values (Table 1). The differences in roughness between samples are in the nanometric scale; R_z mean values ranged from 7.3 to 3.7 μm while R_a mean values ranged from 200 to 530 nm on the tested substrates (Figure 2). Indeed, these obtained values are close to previously reported optimal values for cell adhesion.^{47, 48}

Cells cultured for 24 h were spread and with a well-organized cytoskeleton on all materials (Figure 5). However, whilst osteoblasts were mostly branched on Z-0Ti, Z-25Ti and Z-50Ti, they were round-shaped on Z-75Ti and Z-100Ti substrates, disclosing an earlier adhesion stage. In addition, the cytoplasm extension was significantly enhanced on Z-0Ti ($p < 0.01$) and on Z-Ti

cermets containing 3Y-TZP ($p < 0.05$), either 24 or 48 h after seeding. After 48 h of culture, cells had a more advanced adhesion step revealed by more complex spindle morphologies and long filopodia establishing intercellular contacts, mainly on Z-0Ti and Z-Ti cermets (Figure 6(a)). This agreed with a strong signal of vinculin, particularly on Z-Ti cermets (Figure 5(b)). As expected from an osteoblastic phenotype, cell circularity decreased on the set of materials along the culture time; although faster on Z-0Ti ($p < 0.01$) and on Z-Ti cermets ($p < 0.05$) than on Z-100Ti specimens (Figure 6(b)).

We were able to manufacture materials containing nanostructured 3Y-TZP by using SPS technology. Other investigations have also described a higher osteoblastic activity on nanosized materials, comparing to those of micrometric structures.^{49, 50} The nanostructured surfaces possess unique properties that influence cell adhesion directly by affecting the cell–surface interactions and indirectly by influencing the protein–surface interactions.⁵¹ These specific effects of nanotopography enhancing cell behaviour have been demonstrated using different cell types like epithelial cells, fibroblasts, myocytes and osteoblasts⁵².

Cell proliferation and ALP activity assay for osteoblastic differentiation. Figure 7(a) shows that the number of SaOS-2 cells quantified by the LDH assay increased during the culture time on all tested substrates. At the beginning of the proliferation assay (1 and 3 days), the number of cells was enhanced on materials with high percentage of 3Y-TZP. This trend was maintained at longer culture times (7 and 14 days) with the exception that statistically higher cell numbers were found on Z-75Ti surfaces at both time points.

Alkaline phosphatase (ALP), osteocalcin, osteopontin or type I collagen are often used to analyze osteoblastic differentiation.^{28, 53} Among them, ALP activity is an early-marker of differentiation because it provides phosphate ions for the hydroxyapatite formation.²⁹ Thereby, early differentiation was studied by measuring the ALP activity in SaOS-2 cells cultured on the

different substrates using osteogenic medium. As shown in Figure 9(b), this enzymatic activity increased with time on all the series studied. However, cytoplasmic ALP activity was higher on 3Y-TZP containing surfaces at earlier culture times, whereas it was similar in all samples at 14 days except for Z-75Ti substrates, which showed the statistically significant highest value.

In agreement with previous results, proliferation and differentiation increased simultaneously on 3Y-TZP containing materials at earlier culture times.⁵⁴ Interestingly, Z-75Ti cermet and as a consequence of being the specimens with lower average values of R_a (200 nm) and R_z (4 μ m), as well as with higher hydrophilic surface character (54° of DI-water CA), led the highest proliferation and differentiation levels at 14 days. Thereby, similar manufacturing methods led to different surface properties that induce different osteoblastic behaviour as a consequence of a different nature in the materials. Furthermore, by combining dissimilar properties of ceramic materials (3Y-TZP) and metal (Titanium) into composites (3Y-TZP/Ti cermets) a better biological response was found comparing to the pure counterparts.

Conclusions

SPS is consolidated as a promising technique to obtain 3Y-TZP/Ti stable cermets for biomedical applications. No cytotoxic effects were found on osteoblasts cultured onto the cermets with different compositions. In general, higher values of adhesion, spreading, proliferation and earlier differentiation were found in materials containing a nanostructured 3Y-TZP phase as compared to plain (micrometric) titanium. Moreover, Z-Ti specimens had a combination of surface properties, in fact sub-micrometric roughness and moderately hydrophilic character that stimulated highest values of osteoblast proliferation and differentiation at longer culture times. This research contributes therefore to increase the knowledge on the biological interactions that occur on materials with clinical relevance, such as zirconia and titanium, and their composites.

Acknowledgements

E. Fernandez-Garcia acknowledges CSIC for the concession of a JAE-PreDoc 2010. C.F. Gutierrez-Gonzalez acknowledges CSIC for the concession of a JAE-Doc 2009. Parts of this work were carried out in the Photoelectron Spectroscopy, UV-Visible and Infrared Unity of the Scientific Technical Services (SCTs), University of Oviedo.

Funding

This work was supported by the Spanish Ministry of Science and Innovation (MICINN) under the project [MAT2009-14542-C02] and by the Spanish Ministry of Economy and Competitiveness (MINECO) under the project [MAT2012-38645] and [MATBIO-INTERFACES-MAT2012-38645]. Support for the research by M. P. Ginebra was received through the prize “ICREA Academia” for excellence in research, funded by the Generalitat de Catalunya.

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FIGURE CAPTIONS

Figure 1. Microstructure: FE-SEM image revealing nanofeatures in pure 3Y-TZP (Z-0Ti).

Figure 2. Roughness: interferometric 3-D images and mean $\pm$ SD values of roughness parameters. Every substrate had isotropic roughness with sub-micrometric average values. Higher R_a values matched with higher RSA and S_{Al} values.

Figure 3. Chemical composition studied from XPS survey scans acquired from the diverse substrates.

Figure 4. Cytotoxicity: Cell viability (%) higher than 80% on all materials dismissed a potential cytotoxic effect.

Figure 5. Immunostaining: **(a)** the red-fluorescence of F-actin was visualized by fluorescent microscopy and disclosed that osteoblasts were more elongated on materials containing 3Y-TZP both 24 and 48 h after seeding (Scale bar=50 μ m); **(b)** the green-fluorescence of vinculin forming part of focal adhesions was assessed by confocal microscopy 48 h after plating revealing large areas of this protein on materials containing 3Y-TZP (Scale bar=25 μ m).

Figure 6. Cell adhesion: quantification of cell area **(a)** and circularity **(b)** of osteoblasts cultured for 24 and 48 h. Data are mean $\pm$ the corresponding standard error. In **(b)**, circularity equal to one implies a perfect circle, whilst zero implies a straight line. At each time: *(a)* compares to Z-0Ti, *(b)* compares to Z-25Ti, *(c)* compares to Z-50Ti, *(d)* compares to Z-75Ti and *(e)* compares to Z-100Ti; $p<0.05$ (1 letter) and $p<0.01$ (2 letters).

Figure 7. Time course study of osteoblast proliferation **(a)** and osteogenic differentiation **(b)**: data are mean $\pm$ SEM values ($n=3$). At each incubation time, *(a)* indicates differences from Z-0Ti, *(b)* differences from Z-25Ti, *(c)* differences from Z-50Ti, *(d)* differences from Z-75Ti and *(e)* differences from Ti; $p<0.05$ (1 letter) and $p<0.01$ (2 letters).

TABLES

Raw Materials (vol. %)	YT-TZP/Ti composites (Z-Ti)				
	Z-0Ti	Z-25Ti	Z-50Ti	Z-75Ti	Z-100Ti
3Y-TZP	100	75	50	25	0
Ti	0	25	50	75	100

Table 1. The diverse 3Y-TZP/Ti composites (Z-Ti) involved in the study are performed by varying the relative amount (vol.%) of 3Y-TZP and Ti raw materials.

CA (°)	Surface				
	Z-0Ti	Z-25Ti	Z-50Ti	Z-75Ti	Z-100Ti
DI-water	88.6±1.2	86.8±3.3	88.1±4.5	54.3±1.3	86.6±3.0

Table 2: Mean ± SD results of advancing contact angle, CA, of Distilled (DI) water sessile drop on the studied surfaces

Surfaces	Atom (%)				
	O 1s	Ti 2p	C 1s	Zr 3d	Y 3d
Z-0Ti	28.9±3.0	-	44.8±6.2	24.5±2.7	1.8±0.5
Z-25Ti	28.5±1.7	2.3±1.3	51.6±7.2	16.7±4.2	0.9±0.3
Z-50Ti	33.5±3.1	5.5±1.1	52.7±1.5	7.9±0.4	0.3±0.1
Z-75Ti	36.2±1.3	7.6±0.7	52.0±1.9	3.5±0.2	-

Z-100Ti	36.9±0.1	14.5±3.2	52.2±2.0	-	-
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Table 3: X-ray photoelectron spectroscopy atomic composition

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7