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EFFECT OF 64S BIOGLASS ADDITION ON SINTERING KINETIC, FLEXURAL STRENGTH AND OSTEOBLAST CELL RESPONSE OF Y-TZP CERAMICS

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ABSTRACT

3 mol% yttria-partially stabilized zirconia (Y-TZP) with 10.5 and 19.9 vol% 64S bioglass compacts were sintered at different temperatures up to 1500 ^{0}C. The influence of 64S glass addition on the sintering kinetic, flexural strength and osteoblast cell response of Y-TZP ceramics was investigated. The addition of 64S glass increased the initial sintering rate through the decrease in the activation energy and the increase in the order of diffusion with respect to those previously reported for

Y-TZP. Y-TZP at 1500 ^{0}C exhibited the highest flexural strength. Within Y-TZP ceramics with 64S additions, a maximum flexural strength occurred for 10.5 vol% 64S at 1400 ^{0}C, its flexural strength was able to approach that of Y-TZP at 1500 ^{0}C. The polished sintered surfaces became rougher as the 64S content increased. Cell viability experiments on the less nano-roughness Y-TZP and Y-TZP with 10.5 vol% 64S surfaces revealed their good biocompatibility; on the contrary, the high level of nano-roughness of Y-TZP with 19.9 vol% 64S significantly reduced cell survival. However, the matrix mineralization was not adversely affected by the surface roughness; larger amounts of calcium phosphate phases on Y-TZP-19.9 vol% 64S surfaces appeared to promote the osteogenic potential of UMR-106 cells.

Keywords: Y-TZP- (64S) bioglass compacts, sintering kinetic, flexural strength, osteoblast cell response.

1. INTRODUCTION

Y-TZP ceramics with bioglass additions, have received increasingly attention from scientific and medical communities as dental implant materials and dental restorations owing to their high strength, abrasion resistance, good biocompatibility and aesthetics[1].

The incorporation of frit glasses, that form a viscous flow during the sintering process, is an attractive way to promote the Y-TZP densification[2,3]. However, there is a lack of studies that evaluate the densification behaviour and microstructural development of Y-TZP containing a refractory glass composition, which does not melt at the sintering temperatures, as an additive. In this sense, we have

investigated[4] a bioglass having the above mentioned characteristics, named 64S, as an additive for Y-TZP. The microstructural observation of Y-TZP-64S compacts sintered at 1300-1500 ^{0}C by scanning electron microscopy (SEM), has shown that secondary phases ($Ca_2P_2O_7$ and $ZrSiO_4$) were uniformly distributed in the fine grain matrix of zirconia. The presence of low amounts of calcium phosphate phases would have the potential to improve the osteoblast cell response of Y-TZP ceramics, while maintaining their good mechanical performance.

The possibility of inducing the tetragonal (t) to monoclinic (m) zirconia transformation by a mechanical stress has been successfully used to obtain a reinforcing mechanism in ZrO_2- based ceramics[4]. Apart from the toughening mechanism, the t-m ZrO_2 transformation can be induced by hydrothermal degradation in the humid environment of the oral cavity[5]. Therefore, another aspect to be considered in Y-TZP ceramics is the hydrothermal degradation or ageing which is accompanied by intergranular microcracking and results in the loss of mechanical properties. It is expected that the secondary phases dispersed in the ZrO_2 matrix decrease the m-ZrO_2 surface content and also act as a barrier between ZrO_2 grains retarding the propagation of the transformation. Biological and mechanical properties as well as hydrothermal degradation are of great importance because of their influence on the clinical success of this kind of bioceramics.

Y-TZP with 10.5 and 19.9 vol% 64S compacts were sintered at different temperatures up to 1500 ^{0}C. Our previous study[6] has pointed out that the 64S glass addition markedly increased the Y-TZP solid state sintering rate. In this work the effect of 64S additive on the Y-TZP sintering kinetic is studied. The initial sintering

kinetic of Y-TZP powder has been extensively investigated by many researchers: Young and Cutler[7] have measured the Y-TZP shrinkage at constant rates of heating (CRH) and applied sintering-rate equations to their data to estimate the activation energy at the initial sintering stage. Matsui et al.[8] have investigated the sintering kinetic at isothermal shrinkage of fine zirconia powders. The influence of SiO_2 and Al_2O_3 additions on the initial sintering step of Y-TZP powder has also been studied by Matsui et al.[9-11]. However, the effect of 64S glass addition on the initial sintering kinetic of Y-TZP has not been reported in the literature.

Other aims of this work are to investigate the flexural strength and osteoblast cell response of sintered Y-TZP- 64S ceramics and compare them with those of Y-TZP. It has been well documented[12, 13] that cells are sensitive to modifications in the surface topography. Topographical changes at the nanometer scale have shown promising results both in vitro and in vivo[14-16]. Roughness at the nanometer scale was created in the present work by polishing the ceramics. It is expected that variations in the monoclinic (m)- ZrO_2 and secondary phases contents in Y-TZP with different amounts of 64S glass will lead to changes in surface roughness of polished samples on nanometer scale, and consequently on the osteoblast cell response.

In this work, the activation energy and diffusion mechanism of Y-TZP with 10.5 vol% 64S addition at the initial sintering stage were studied by CRH techniques. The influence of 64S content and sintering temperature on the flexural strength of the compacts was evaluated. Finally, the in vitro biocompatibility and osteogenic cell differentiation of polished sintered Y-TZP and Y-TZP- 64S surfaces were assessed using an osteoblastic cell culture. The hypothesis to be tested was that the addition

of low amounts of 64S glass to Y-TZP may increase the sintering rate and promote the osteoblast cell response, while maintaining acceptable flexural strength values. Therefore, the addition of 64S glass may have a positive effect not only in the manufacture process of sintered Y-TZP ceramics but also in the biological behaviour of dental implant abutments and dental restorations.

2. EXPERIMENTAL PROCEDURE

2.1. Raw materials, powder processing and characterization

The starting powder was a commercial 3 mol% yttria- partially stabilized zirconia without alumina (Y-TZP) (Saint-Gobain ZirPro, Chine) with a specific surface area (S_g) of 7.0 m^2/g and more frequent particle diameters of 0.14 and 0.48 µm (measured using a Mastersizer 2000, Malvern Instruments, UK). The synthesis of the CaO- P_2O_5- SiO_2 glass with composition: 64% SiO_2, 26% CaO and 10% P_2O_5 (based on mol%),named 64S in this work, was performed by the sol-gel method; details of the processing procedure are reported elsewhere[6]. Milling of the glass powder was done by attrition milling in isopropyl alcohol using 1.6 mm zirconia balls during 4 h, then the powder was dried at 60 ^{o}C for 24 h. The 64S glass presented a mean particle diameter of 3.0 µm and its measured density was 2.70 g/cm^3.

Y-TZP, Y-TZP with 10.5 vol% 64S (Y-TZP10) and Y-TZP with 19.9 vol% 64S (Y-TZP20) ceramics were investigated in this study. 43 vol% aqueous Y-TZP and Y-TZP-64S suspensions were prepared by dispersing the powders in deionized water with the optimum deflocculant (Duramax D 3500, Rohm & Haas, Philadelphia PA) concentration using an ultrasonic bath; when it was necessary, the pH was manually adjusted to be maintained at 9 with a diluted aqueous ammonia solution. Slips were

cast in plaster molds into 1.85 cm diameter disks; the consolidated disks were air dried at room temperature followed by 24 h at 100 ^{0}C. The resulting green compacts were sintered in air at 1100-1500 ^{0}C during 2h with a heating/cooling rate of 10 ^{0}C/min.

Crystalline phases were characterized by X-ray diffraction (Bruker, D2 Phaser, with Kα: Cu as incident radiation and Ni filter); the equipment was operated at 30 kV and 10 mA and the scanning was performed with a step of 0.04° and 2.5 s per step in the 10°-80° 2θ range. In order to perform phase quantification, the XRD patterns were analysed with PROGRAM FullProf.2k (Version 5.80 - May 2016-ILL JRC) which is a multipurpose profile-fitting program, including Rietveld refinement method[17,18].

The Archimedes method with distilled water as the immersion liquid was employed to determine the bulk density of the sintered compacts; a theoretical density of 6.05 g/cm^3 was used to calculate the relative density of Y-TZP. The theoretical density of the sintered Y-TZP- 64S compacts at each temperature was calculated from the density values of the different phases and their respective volume fraction.

The sintered samples were diamond polished down to 3 μm and thermally etched at a temperature 50 ^{0}C below each sintering temperature for 30 min. The average zirconia (ZrO_2) grain sizes of the sintered compacts were measured from SEM images using Image J software according to the linear interception method; the given grain sizes averaged at least 200 grains.

The three-point flexural strength of sintered samples was measured on (45 x 4 x 3 mm^3) size bars using a universal testing machine (INSTRON, 4483) according to the ASTM C1161-13 standard test method. At least ten measurements of flexural strength were tested for each sample and the results are reported as mean ± standard deviation.

The morphology of the phases was observed by SEM (FEI, Quanta 200); compositional analysis was performed by energy dispersive x-ray spectrometry (EDS). Polished sintered samples were characterized by atomic force microscopy (AFM) (Nanoscope V; Bruker, Santa Barbara, CA) in tapping mode, using silicon tips (budget sensor; spring constant, 40N/m; resonance frequency, 300 kHz) and a scan rate of 1 Hz; Nanoscope 7.30 and Nanoscope Analyis 1.5 softwares were employed to obtain the images (Bruker) with acquisition of 512 x 512 pixels per image. The roughness of the polished surfaces was evaluated in terms of the average (R_a) values. R_a of five areas of 5 μm x 5 μm in the polished surfaces was recorded for each sample and the reported values are the mean ± standard deviation of the five determinations.

Contact angle measurements were performed using a goniometer (Ramé-Hart 290-V1) equipped with a digital camera. Ultra-pure water (MilliQ$^®$) was used as the wetting liquid with a drop size of 5μl. Contact angles of the air-water-substrate interface were measured for each group and the values are expressed as mean ± standard deviation.

2.2. CRH measurements and kinetic analysis

For the CRH measurements, 43 vol% aqueous Y-TZP10 suspensions with the optimum amount of deflocculant were cast into cylindrical disks (5 mm in diameter and 6 mm in length). The shrinkage of the compacts as a function of the sintering temperature was determined by hot-stage microscopy (Linseis). The shrinkage measurements were carried out from room temperature to 1450 ^{0}C in air at heating rates of 5, 10 and 15 ^{0}C/min. The initial thermal expansion of the samples was corrected from the measured shrinkages using the thermal expansion coefficient of Y-TZP[10]. The shrinkage percentage of the diameter axis was nearly the same to that of the length axis; assuming isotropic shrinkage of the compact the density (ρ) at a temperature (T) can be calculated by:

$$\rho \ (T) = \left(\frac{L_f}{L(T)}\right)^3 \rho_f \qquad (1)$$

Where ρ_f is the final density measured by the Archimedes method, and L (T) and L_f are the length at T and the final length, respectively. A theoretical density of 5.65 g/cm^3 was used to calculate the relative density of Y-TZP10[6].

The sintering rate equation at the initial sintering stage is expressed as[8]:

$$\frac{d}{dt}\left[\left(\frac{\Delta L}{Lo}\right)^{1/n}\right] = \frac{K \gamma s \, \Omega \, D}{k \, T \, a^p} \qquad (2)$$

Where K is a numerical constant, γs is the surface energy, Ω is the atomic volume, D indicates the diffusion coefficient, k is the Boltzmann constant, a indicates the particle radius, n and p parameters are the order which depend on the diffusion mechanism; p value for grain-boundary diffusion (GBD) is 4 and that for volume diffusion (VD) is 3. Lo and ΔL= (Lo-L) are the initial length and the length change of the sample, respectively, and t is the time. Equation (2) satisfies the initial sintering stage (fractional shrinkage < 4%).

The following sintering rate equation has been derived from Eq. (2) by Wang and Raj[19]:

$$\ln\left[T\left(\frac{dT}{dt}\right)\left(\frac{d\rho}{dT}\right)\right] = \frac{Q}{RT} + \propto \qquad (3)$$

α can be expressed as follows:

$$\alpha = \ln[f(\rho)] + \ln\left[\frac{K\,\gamma s\,\Omega\,Do}{k}\right] - p\ln a \qquad (4)$$

$f(\rho)$ is a function of density, Do is the pre-exponential term of the diffusion coefficient given by:

$$D = Do\, e^{-Q/RT} \qquad (5)$$

Q is the activation energy and R is the gas constant. Q can be calculated from the slope (S) of the Arrhenius-type plot of $\ln\left[T\left(\frac{dT}{dt}\right)\left(\frac{d\rho}{dT}\right)\right]$ versus 1/T (Eq. 3) at the same density, according to the following equation:

$$Q = -RS \qquad (6)$$

On the other hand, the sintering rate equation (7) has been derived from Eq. (2) by Matsui[20]:

$$\frac{d\left(\frac{\Delta L}{Lo}\right)}{dT} = \left(\frac{K\,\gamma s\,\Omega\,Do\,R}{k\,a^p\,c\,Q}\right)^n \cdot \left(\frac{nQ}{R\,T^{2-n}}\right) \cdot e^{-n\,Q/RT} \qquad (7)$$

Where c $\left(\frac{dT}{dt}\right)$ is the heating rate; Eq. (7) is similar to that derived by Young and Cutler[7] for GBD (n=1/3) and VD (n=1/2). The apparent activation energy (nQ) can be calculated from the slope (S$_1$) of the Arrhenius-type plot of $\ln\left[T^{2-n}\,\frac{d\left(\frac{\Delta L}{Lo}\right)}{dT}\right]$ as a function of 1/T, in this way nQ is given by:

$$n\,Q = -RS_1 \qquad (8)$$

The order n is between 0.31 and 0.50 (depending on the diffusion mechanism) and it can be determined combining Eqs. (6) and (8):

$$n = \frac{nQ}{Q} = \frac{S1}{S} \qquad (9)$$

Matsui et al.[11] have reported that the log ($\Delta L/Lo$) -log t plots at different constant temperatures showed linear relations in the initial sintering stage (shrinkage range <4%); Moriyoshi and Komatsu[21] have demonstrated that the plot of log ($\Delta L/Lo$)- log t at the initial sintering step is linear when grain growth does not occur simultaneously. Thus, in the shrinkage range <4% grain growth is expected to be negligible. As a consequence in the present work, the Q and n values were determined in the fractional shrinkage <4%, which satisfies the initial sintering condition without grain growth.

A summary of all the variables measured in sections 2.1 and 2.2 on the different groups is given in table 1.

2.3. Biological assays

2.3.1. Cell culture

Osteoblastic cells (UMR-106, CRL-1661, American Type Cell Collection-ATCC) were cultivated in growth media (GM), formed by D-MEM (Dulbecco's Modified Eagle Medium, Invitrogen-Life Technologies, NY, USA), 10% fetal bovine serum (Gibco, NY, USA) and 1% penicillin-streptomycin (10,000 U/mL, Gibco). Cultures were harvested with 1 Mm ethylenediamine tetra acetic acid (EDTA) (Gibco) and 0.25% trypsin (Gibco) after subconfluence. Then, subcultured cells were

plated in 24-well culture plates at a cell density of 2×10^4 cells on the discs and cultured in osteogenic medium (OM), constituted by GM with 50 µg/mL ascorbic acid (Gibco) and 7 mM β-glycerophosphate (Sigma-Aldrich, MO, USA), for periods of up to 7 days. Cultures were kept in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C.

2.3.2. Cell morphology

At day 3, cell morphology was examined by direct fluorescence to detect cell nuclei and the actin cytoskeleton. Cells were fixed at room temperature using 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) and permeabilized with 0.5% Triton X-100 in PB for 10 min. Cells were then processed for fluorescence labelling using Alexa Fluor 594 (red fluorescence)-conjugated phalloidin (1:200, Molecular Probes, OR, USA) to identify the actin cytoskeleton; and 300 nM 40,6-diamidino-2-phenylindole, dihydrochloride (DAPI, Molecular Probes) to label the cell nuclei. The samples were observed under epifluorescence, using a Zeiss AxioImager M2 microscope (Carl Zeiss, Germany) outfitted with an Axio Cam MRm digital camera (Carl Zeiss). Adobe Photoshop CS5.1 software (Adobe Systems, CA, USA) was used to process the digital images.

2.3.3. MTT assay

At day 3, cell viability was evaluated by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich) assay. The cultures were incubated with 10% MTT (5 mg/mL) in culture medium at 37 °C for 4 h. To perform the quantitative analysis, 1 mL acid-isopropanol (0.04 N HCl in isopropanol) was added to each well, and the solution was transferred to a 96-well plate (Corning Inc.).

The optical density was read on the plate reader (µQuanti, BioTek Instruments Inc., VT, USA) at 570–650 nm and data were expressed as absorbance.

2.3.4. Matrix mineralization

At day 7, cultures grown on the discs were fixed in 10% formalin at room temperature for 2h, dehydrated and stained with 2% Alizarin Red S (Sigma) (pH 4.2) for 10 min. Macroscopic images of the cultures were digitally obtained with a high-resolution camera (Canon EOS Digital Rebel, 6.3 megapixels, with macro lens EF100 f/2.8). A colorimetric method[22] was used to detect the calcium content; briefly, 280 µl of 10% acetic acid was added to each well and the plate was incubated at room temperature under shaking for 30 min. This solution was vortexed for 1min, heated to 85 ^{o}C for 10 min and transferred to ice for 5 min. The slurry was centrifuged at 13,000 g for 15 min and 100 µl of the supernatant was mixed with 40 µl of 10% ammonium hydroxide. The optical density was read at 405 nm and data were expressed as absorbance.

2.3.5. Statistical analysis

The normality and homoscedasticity of the data were assessed by the Shapiro-Wilk test and Levene's mean test, respectively, using the Sigma Plot software (Systat Software Inc., San Jose, CA). The data obtained with the MTT assay and the Alizarin Red S extraction were then analysed by one-way ANOVA and reported as mean ± standard deviation. The level of significance (p) was set at 0.05 for all comparisons.

3. RESULTS AND DISCUSSION

3.1. Densification behaviour and grain growth

Table 2 shows the change in relative densities of Y-TZP, Y-TZP10 and Y-TZP20 with sintering temperature. The relative density of Y-TZP10 and Y-TZP20 were higher than that of Y-TZP at 1100-1400 $^{\circ}$C. Y-TZP10 and Y-TZP20 reached 99% of the theoretical density at 1400 ^{0}C, whereas Y-TZP attained ~ 98% of the theoretical density at 1500 ^{0}C. Thus, the addition of 64S glass accelerated the densification of Y-TZP. Y-TZP10 compacts were selected to investigate the effect of 64S glass addition on the initial sintering kinetic. The activation energy and the diffusion mechanism at the initial sintering step of Y-TZP10 were estimated using the CRH method, and compared with those of Y-TZP which has been extensively investigated[7, 8, 10, 23].

Figure 1 presents the ZrO_2 average grain size in the different compacts as a function of the 64S glass content at 1250-1500 ^{0}C for 2 h. At 1250 ^{0}C, the addition of 64S glass had no effect on the ZrO_2 grain growth. At 1300 ^{0}C, the ZrO_2 average grain size remained virtually unchanged up to 10.5 vol% 64S, an increase in the amount of 64S over 10.5 vol% slightly increased the ZrO_2 grain size. At 1400-1500 ^{0}C, the ZrO_2 average grain size gradually increased up to 10.5 vol% 64S, over 10.5 vol% a rapid increased was found. At the sintering hold time of 2 h, 19.9 vol% 64S addition enhanced the ZrO_2 grain growth at temperatures > 1250 ^{0}C; whereas, the ZrO_2 grain size increased by the presence of 10.5 vol% 64S at temperatures higher than 1300 ^{0}C. The ZrO_2 average grain size of Y-TZP10 at 1250-1300 ^{0}C was about 0.35 μm.

3.2. CRH shrinkage analysis of Y-TZP10

The shrinkage of Y-TZP10 green compact during heating at 5, 10 and 15 ^{0}C/min heating rates was measured; figure 2a shows the shrinkage of Y-TZP10 as a function of temperature at 5 ^{0}C/min heating rate. The compact shrinkage started at ~ 1100 ^{0}C and it was < 4% at temperatures lower than 1280 ^{0}C. As it was mentioned in section 3.1, at temperatures lower than 1300 ^{0}C the addition of 10.5 vol% 64S had no effect on the ZrO_2 grain growth (fig. 1).

Figure 2b shows the relative density (determined using Eq. 1) versus temperature curves for Y-TZP10 at 5, 10 and 15 ^{0}C/min heating rates; as the heating rate increased, the curves shifted to a higher temperature. The densification rate ($\frac{d\rho}{dT}$) at 5,10 and 15 ^{0}C/min heating rates was calculated using the relative density vs. T curves in fig. 2b. As an example, the densification rate vs. temperature curve of Y-TZP10 measured at 5 ^{0}C/min is presented in figure 2c; the maximum densification rate was found at about 1360 ^{0}C.

Applying Eq. (3) to the above results, the activation energy at the initial sintering stage was determined as follows: at the same density both T and $\frac{d\rho}{dT}$ were determined at each heating rate ($\frac{dT}{dt}$); the obtained values were plotted as ln [T ($\frac{dT}{dt}$) $\left(\frac{d\rho}{dT} \right)$] as a function of 1/T in figure 3. The analysis was done in the relative density range of 0.51-0.54 (fractional shrinkage range < 4%) which corresponds to the initial sintering step without grain growth. The Q values at 0.51-0.54 relative densities were calculated from the slope of the straight lines (correlation

coefficient=0.99) in the Arrhenius-type plot (fig. 3), and an average Q value of 466 ± 10 KJ/mol for Y-TZP10 was obtained.

Wang and Raj[23] measured an activation energy of 615 KJ/mol for sintering of 2.8 mol% Y-TZP using the CRH method. Matsui[10] also reported that the Q value for sintering of 3 mol% $Y_2O_3 - ZrO_2$ powder with S_g of 6.7 m^2/g (similar to the S_g value of the Y-TZP powder used in this study) measured by the CRH analysis was 647 KJ/mol. Thus, the Q value determined in the present work for Y-TZP10 (466 KJ/mol) was significantly smaller compared to that previously reported for Y-TZP. There was a clear indication that the addition of 10.5 vol% 64S to Y-TZP powder decreased the activation energy at the initial sintering stage.

The reduced activation energy by the presence of 64S glass suggested that a change in the diffusion mechanism might have occurred. From Eq. (7), the diffusion mechanism was determined as follows: the nQ values were calculated from the slopes of $\ln \left[T^{2-n} \frac{d(\frac{\Delta L}{Lo})}{dT} \right]$ vs. 1/T for GBD (n=1/3) and VD (n=1/2), using the shrinkage curve of each heating rate in the fractional shrinkage range < 4%. The plots at 5 ^{0}C/min are shown in figure 4, as an example; linear relations were found in both plots. In a similar manner, linear relations for the plots at 10 and 15 ^{0}C/min heating rates (not shown) were obtained. An average nQ value of 224 was calculated from the slopes of the GBD and VD-type plots in figure 4. Then, the n value was determined by Eq. (9) using the average values of nQ and Q, in this way an n value of 0.48 for Y-TZP10 was obtained. The value of n previously reported for

Y-TZP, with a similar S_g to that of the Y-TZP powder used in this study, was 0.32-0.33[10]. The n value of GBD varies from 0.31 and 0.33 and that of VD from 0.40 and 0.50[9]. Thus, the above mentioned results indicated that the diffusion mechanism of Y-TZP10 was VD in contrast with GBD previously determined for Y-TZP. The addition of 64S glass changed the diffusion mechanism at the initial sintering step from GBD to VD and decreased the activation energy of diffusion.

The effect of 64S glass addition on the sintering rate can be explained considering the integral type of Eq. (7) given by:

$$\frac{\Delta L}{Lo} = \left(\frac{K \gamma s \, \Omega \, Do \, R}{k \, a^p \, c \, Q}\right)^n . T^n . e^{-n \, Q/RT} \quad (10)$$

Taking into account that the nQ value of 224 estimated for Y-TZP10 was not much different from 220 reported for Y-TZP[10], the increase in $\frac{\Delta L}{Lo}$ in Eq. (10) and consequently in the sintering rate of Y-TZP10, at a constant nQ and T, can be attributed to the increase in n and the decrease in both Q and p in the $\left(\frac{K \gamma s \, \Omega \, Do \, R}{k \, a^p \, c \, Q}\right)^n T^n$ pre-exponential term. Thus, the 64S addition changed the diffusion mechanism from GBD to VD, and markedly increased the densification rate through the decrease in both Q and p and the increase in n of VD.

We have previously studied[6] the thermal decomposition of the 64S glass at 950-1500 ^{0}C, the major phase was SiO_2 and $Ca_3(PO_4)_2$ was present as minor phase. Ikuhara et al.[24] investigated the sintering behaviour of 2.5 mol% Y-TZP with addition

of SiO$_2$ particles by transmission electron microscopy; they found that the SiO$_2$ particles were adsorbed into the ZrO$_2$ grains at 1300 ^{0}C and segregation of Y^{+3} and Si^{+4} ions at grain boundaries was observed. Matsui[9] demonstrated that the addition of a small amount of SiO$_2$ to Y-TZP powder changed the diffusion mechanism from GBD to VD at the initial sintering stage, and markedly increased the sintering rate of Y-TZP; he reported that the increase of sintering rate by SiO$_2$ addition was related to the segregated dissolution of SiO$_2$ in Y-TZP. In the present work, the main SiO$_2$ phase produced during 64S annealing was expected to increase the Y-TZP sintering rate at the initial stage. The diffusion mechanism of VD at the initial sintering stage of Y-TZP10 could be explained considering the dissolution of a small amount of SiO$_2$, produced by thermal decomposition of 64S glass, in Y-TZP grains.

3.3 Characterization of sintered samples and flexural strength

According to the Rietvield analysis of samples sintered at 1300-1500 ^{0}C (table 3), the major crystalline phase was tetragonal (t)- ZrO$_2$ accompanied by lower amounts of m- ZrO$_2$, and secondary phases (Ca$_2$P$_2$O$_7$ and ZrSiO$_4$) in Y-TZP10 and Y-TZP20. ZrSiO$_4$ was formed by a reaction between ZrO$_2$ and most of the SiO$_2$ produced by 64S annealing. The sintering temperature did not affect the development of Ca$_2$P$_2$O$_7$ and ZrSiO$_4$ phases, the amount of these phases only varied with the 64S glass content; on the contrary, the m- ZrO$_2$ content was dependent on both the 64S glass content and the sintering temperature (table 3). The total quantity of secondary phases (Ca$_2$P$_2$O$_7$ + ZrSiO$_4$) increased from ~8.4 to ~19.4 vol% with increasing the 64S glass content from 10.5 to 19.9 vol%.

At 1300 ^{0}C, the m- ZrO_2 content hardly changed with increasing 64S glass. The m- ZrO_2 content at 1400 ^{0} C slightly increased up to 10.5 vol%, for 19.9 vol% 64S a greater t-m transformation was found. It is generally recognized that the ZrO_2 grain size controls the stability of the tetragonal phase[25]; the increase in the ZrO_2 grain size enhances the spontaneous t-m transformation after sintering. As the 64S glass content increased from 0 to 10.5 vol% a scarcely increased in the ZrO_2 average grain size at 1400 ^{0}C from 0.42 to 0.49 µm was found; however, a more pronounced ZrO_2 grain growth from 0.49 to 0.70 µm occurred as the 64S glass content increased from 10.5 to 19.9 vol% leading to a lesser stability of the tetragonal phase (fig. 1 and table 3). The increase in the sintering temperature from 1400 to 1500 ^{0}C enhanced the ZrO_2 grain growth, thereby a low retention of t- ZrO_2 was found at 1500 ^{0}C in Y-TZP with 64S additions. A 64S glass content of 19.9 vol% resulted in a substantial increase in the average grain size of ZrO_2 (~1µm) at 1500 ^{0}C, consequently ~ 21 vol% of the ZrO_2 was presented as the monoclinic phase (fig. 1 and table 3). The m- ZrO_2 content of Y-TZP at 1500 ^{0}C (2.2 vol%) was similar to that of Y-TZP10 at 1400 ^{0}C (2.8 vol%) due to their similar ZrO_2 average grain sizes (fig. 1 and table 3).

Figure 5 shows the flexural strength of the sintered samples as a function of 64S glass content at 1300-1500 ^{0}C. At 1300 ^{0}C, the presence of 64S glass resulted in an increase in the flexural strength. This behaviour could be attributed to the enhanced densification of Y-TZP10 and Y-TZP20 compacts compared to Y-TZP at 1300 ^{0}C (table 2); thus, the strength was determined by the flaw size.

At 1400 ^{0}C, a maximum flexural strength was found at 10.5 vol% 64S, reaching a value of ~ 750 MPa. The addition of 10.5 vol% 64S glass increased the sintered density at 1400 ^{0}C (table 2), consequently a slightly increased in the flexural strength was found. For 19.9 vol% 64S, the greater t-m transformation after sintering (table 3) reduced the flexural strength; the volume expansion and twining associated with the autotransformation of large ZrO_2 grains during sintering process[26] introduced microcracks resulting in lower flexural strength values of Y-TZP20 compared to Y-TZP and Y-TZP10 at 1400 ^{0}C. As the 64S glass content increased from 0 to 19.9 vol% the flexural strength at 1500 ^{0}C was reduced. Both, the greater t-m transformation and the lower sintered density of Y-TZP20 contributed to its low flexural strength (tables 2 and 3, fig. 5).

Y-TZP at 1500 ^{0}C exhibited the highest flexural strength. Within Y-TZP ceramics with 64S additions, a maximum flexural strength occurred for 10.5 vol% 64S at 1400 ^{0}C, its flexural strength was able to approach that of Y-TZP at 1500 ^{0}C.

The microstructure of sintered Y-TZP with 64S addition will determine its toughening mechanism; in particular the increase in the ZrO_2 grain size and the incorporation of elastic second-phase particles around ZrO_2 grains matrix may promote additional toughening mechanisms, with respect to that of Y-TZP, related to microcracking and to crack deflection-crack pinning by secondary phases. An increase in the resistant to crack extension will be favourable for the mechanical long-term behaviour of dental implant and dental restorations due to their damage tolerance.

3.4. Characterization of polished sintered samples and biological studies

Y-TZP, Y-TZP10-Y-TZP20 samples, nearly fully densified at 1500 ^{0}C and 1400 ^{0}C, respectively, were characterized by AFM. The AFM observations provide insights about the change in the surface relief. The height image, the derivate image (often called the amplitude error in tapping mode) and the phase image can be obtained from the scans. The brighter is the height image, the higher the local surface relief is. Different microstructural features such as, self-accomodating variants and height variation due to the presence of different phases, can be observed in the amplitude error image. The phase image gives information about the presence and distribution of different phases.

Figures 6 (a), (b) and (c) show the height, amplitude error and phase AFM images, respectively, of Y-TZP10 sintered at 1400 ^{0}C. The presence of elongated and smaller rounded particles in the Y-TZP matrix was found in the height and amplitude error images. The EDS analysis of these particles performed by SEM indicated that the elongated particles corresponded to $Ca_2P_2O_7$ and the smaller ones to $ZrSiO_4$ (see the insert in fig. 6b). The $Ca_2P_2O_7$ particles were not clearly differentiated in the phase image (fig. 6c) as they appeared in brown colour; on the contrary, white spots belonged to $ZrSiO_4$ homogeneously distributed within the Y-TZP matrix were clearly observed. These secondary phases produced a higher relief, as a consequence an increase in brightness was evidenced in the height image (fig. 6a).

Figure 7 shows the AFM images of Y-TZP sintered at 1500 ^{0}C (a, d, g), Y-TZP10 sintered at 1400 ^{0}C (b, e, h) and Y-TZP20 sintered at 1400 ^{0}C (c, f, i). The t-m transformation is accompanied by a lattice volume increase which modify the surface relief[27]. The presence of self-accommodating variants in the transformed monoclinic phase is a characteristic microstructural feature of the martensitic transformation[27]. Martensitic variants are clearly visible (arrows) in the amplitude error image of Y-TZP sintered at 1500 ^{0}C (fig. 7g). Self-accommodating variants were observed in some of the largest Y-TZP grains of the different samples (figs. 7d, 7e and 7f). The number of martensitic variants increased with increasing the 64S content; the greater grain size of Y-TZP20 at 1400 ^{0}C with respect to Y-TZP at 1500 ^{0}C and Y-TZP10 at 1400 ^{0}C enhanced the transformability of t- ZrO_2 (fig. 1 and table 3).

The t-m transformation and the secondary phases induce a height increase modifying the AFM contrast (figs. 7a, 7b and 7c). These images became brighter with increasing the 64S content as a result of the higher relief. The modified relief of some ZrO_2 grains (those that had undergone the t-m transformation) and the secondary phases-induced relief led to surface roughness (fig. 7).

Figure 8 shows the average roughnesss (R_a) of Y-TZP at 1500 ^{0}C, Y-TZP10 and Y-TZP20 at 1400 ^{0}C. As the 64S content increased the sample's surface became rougher; a larger increase in surface roughness from ~15 to ~ 30 nm was observed with increasing the 64S content from 10.5 vol% in Y-TZP10 to 19.9 vol% in Y-TZP20, respectively. This was attributed to the greater amount of monoclinic ZrO_2 and secondary phases for 19.9 vol% 64S addition (table 3). The ISO requirements

concerning the surface state of Y-TZP biomedical components[28] establishes a surface roughness up to 30 nm; in this context the Y-TZP-64S samples developed in this study fulfil the requirements of dental ISO standards.

The assessment of the wettability of the surfaces (not shown) indicated that all the sintered samples had hydrophilic responses; the contact angle values obtained for Y-TZP at 1500 ^{0}C, Y-TZP10 and Y-TZP20 at 1400 ^{0}C were 84.9 ± 0.2^{0}, 84.6 ± 0.2^{0} and 84.5 ± 0.3^{0}, respectively; thus, the contact angles of the three samples were similar. This suggested that the increase in the nano-roughness of the polished sintered samples did not produce changes in the surface wettability.

The biological studies were performed on polished sintered samples: Y-TZP at 1500 ^{0}C, Y-TZP10 and Y-TZP20 at 1400 ^{0}C. Figures 9 and 10 show the epifluorescence and the MTT assay, respectively, of UMR-106 osteoblastic cell cultures grown on Y-TZP, Y-TZP10 and Y-TZP20 at 3 days of culture. The cells adhered and spread well on the sample′s surfaces; they exhibited polygonal shapes and cell-cell contacts were observed in some regions. Differences in terms of cell morphology between the samples were not detected (fig. 9); the images showed a lower cell density on Y-TZP20 compared to Y-TZP and Y-TZP10 surfaces. Accordingly, similar MTT values were found for Y-TZP and Y-TZP10, and the addition of 19.9 vol% 64S glass to Y-TZP significantly reduced cell survival (fig. 10).

As it was previously mentioned the water contact angle measured on the polished samples was found to be essentially constant, suggesting that the inhibition of cell proliferation in Y-TZP20 samples was directly related to the increase in

surface roughness. A lesser increase in roughness was found with increasing the 64S content from 0 in Y-TZP to 10.5 vol% in Y-TZP10; however, for 19.9 vol% 64S (Y-TZP20) a larger increase in surface roughness to ~ 30 nm was found (fig. 8). The roughness above which a statistically significant reduction in cell viability took place was approximately 15 nm. Therefore, cell survival was comparable on Y-TZP and Y-TZP10 indicating good biocompatibility, whereas a 64S glass addition over 10.5 vol% affected the growth of UMR-106 cell cultures. Despite that, the matrix mineralization was not adversely affected by the surface roughness (fig. 11); indeed, there was a tendency towards a higher mineralization of Y-TZP20 surfaces compared with Y-TZP and Y-TZP10. It has been well known that there is an inverse relationship between proliferation and differentiation of osteoblastic cells; full expression of the osteoblast phenotype leads to terminal cell cycle exit[29,30], which makes these cells capable to initiate bone matrix production and mineralization. Therefore, one could expect lower viability values in osteoblastic cell cultures showing higher osteogenic potential[31]. The increase in the $Ca_2P_2O_7$ content from ~2.9 to ~6.8 vol% with increasing the amount of 64S glass from 10.5 to 19.9 vol%, respectively (table 3), seemed to play a role in the matrix mineralization process. Indeed, the presence of calcium phosphate phases on the sample's surfaces appeared to promote the osteogenic differentiation and consequently the mineralized matrix production by osteoblastic cells, a finding that has also been shown on calcium phosphate-coated metal surfaces[32].

The surface of the ceramics developed in this study is expected to enhance bone healing and to improve the osseointegration process of dental implants.

CONCLUSIONS

The effect of 64S addition on the sintering kinetic, flexural strength and osteoblast cell response of Y-TZP ceramics was investigated. The addition of 64S glass increased the initial sintering rate through the decrease in the activation energy and the increase in the order of diffusion with respect to those previously reported for Y-TZP. Q and n values of 466 KJ/mol and 0.48, respectively, were determined for Y-TZP10.

Y-TZP at 1500 ^{0}C exhibited the highest flexural strength. Within Y-TZP ceramics with 64S additions, a maximum flexural strength occurred for 10.5 vol% 64S at 1400 ^{0}C, its flexural strength was able to approach that of Y-TZP at 1500 ^{0}C.

The modified relief of some ZrO_2 grains and the secondary phases ($Ca_2P_2O_7$ and $ZrSiO_4$) -induced relief led to surface nano-roughness of polished samples. As the 64S content increased the sample's surface became rougher, a larger increase in nano-roughness was observed with increasing the 64S content from 10.5 to 19.9 vol%; this was attributed to the greater amounts of m- ZrO_2 and secondary phases for 19.9 vol% 64S addition. Cell proliferation experiments on polished sintered Y-TZP and Y-TZP with 10.5 vol% 64S surfaces revealed their good biocompatibility, whereas the highest nano-roughness of Y-TZP with 19.9 vol% 64S surface affected the progression of cell cultures. However, the matrix mineralization was not adversely affected by the nano-roughness; the presence of larger amounts of calcium phosphate phases on Y-TZP with 19.9 vol% 64S surfaces appeared to promote the osteogenic potential of UMR-106 cells.

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FIGURE CAPTIONS

Figure 1: ZrO_2 average grain size as a function of the 64S glass content for different sintering temperatures. The sintering hold time was 2 h.

Figure 2: (a) Shrinkage of Y-TZP10 as a function of temperature at 5 ^{0}C/min heating rate. (b) Relative density versus temperature curves for Y-TZP10 at 5, 10 and 15 ^{0}C/min heating rates, (c) Densification rate $(\frac{d\rho}{dT})$ of Y-TZP10 as a function of temperature at 5 ^{0}C/min.

Figure 3: Plot of ln [T $(\frac{dT}{dt})$ $(\frac{d\rho}{dT})$] as a function of 1/T in the relative density range of 0.51-0.54.

Figure 4: Plot of ln [T^{2-n} $\frac{d(\frac{\Delta L}{L_0})}{dT}$] as a function of 1/T for GBD and VD at 5 ^{0}C/min.

Figure 5: Flexural strength of the sintered samples as a function of 64S glass content at 1300-1500 ^{0}C.

Figure 6: Height (a), amplitude error (b) and phase (c) AFM images of Y-TZP10 sintered at 1400 ^{0}C. A SEM micrograph showing the $ZrSiO_4$ (ZS) and $Ca_2P_2O_7$ (CP) particles in the Y-TZP matrix is presented in the insert of fig. 6b.

Figure 7: AFM images of different samples: Y-TZP sintered at 1500 ^{0}C, height (a), amplitude error (d) (g, arrows indicate martensitic variants); Y-TZP10 sintered at 1400 ^{0}C, height (b), amplitude error (e), phase (h); Y-TZP20 sintered at 1400 ^{0}C, height (c), amplitude error (f), phase (i).

Figure 8: Average roughnesss (R_a) of Y-TZP at 1500 ^{0}C, Y-TZP10 and Y-TZP20 at 1400^{0}C .

Figure 9: Epifluorescence of osteoblastic cell cultures grown on Y-TZP at 1500 ^{0}C, Y-TZP10 and Y-TZP20 at 1400^{0}C at 3 days of culture.

Figure 10: Cell viability of osteoblastic cell cultures grown on Y-TZP at 1500 ^{0}C, Y-TZP10 and Y-TZP20 at 1400^{0}C at 3 days of culture. The asterisk indicates statistical difference (Kruskal-Wallis, $p < 0.05$).

Figure 11: Macroscopic imaging of Alizarin Res S-stained osteoblastic cell cultures grown on Y-TZP at 1500 ^{0}C, Y-TZP10 and Y-TZP20 at 1400^{0}C; and quantification of calcium deposits (matrix mineralization) at 7 days of culture.

Table 1: Summary of all the variables measured in sections 2.1 and 2.2 on the different groups

Variables	Groups
Density	Y-TZP, Y-TZP10, Y-TZP20 at 1100 ^{0}C, 1200 ^{0}C, 1300 ^{0}C, 1400 ^{0}C and 1500 ^{0}C
CRH shrinkage	Y-TZP10 at 5, 10 and 15 ^{0}C/min heating rates
ZrO_2 average grain size	Y-TZP, Y-TZP10, Y-TZP20 at 1250 ^{0}C, 1300 ^{0}C, 1400 ^{0}C and 1500 ^{0}C
Crystalline phases contents	Y-TZP, Y-TZP10, Y-TZP20 at 1300 ^{0}C, 1400 ^{0}C and 1500 ^{0}C
Flexural strength	Y-TZP, Y-TZP10, Y-TZP20 at 1300 ^{0}C, 1400 ^{0}C and 1500 ^{0}C
Average roughness	Y-TZP at 1500 ^{0}C, Y-TZP10 and Y-TZP20 at 1400 ^{0}C
Contact angle	Y-TZP at 1500 ^{0}C, Y-TZP10 and Y-TZP20 at 1400 ^{0}C

Table 2: Relative density of Y-TZP, Y-TZP10 and Y-TZP20 as a function of the sintering temperature.

Temperature (^{0}C)	Y-TZP relative density (%)	Y-TZP10 relative density (%)	Y-TZP20 relative density (%)
1100	53.24	57.64	57.47
1200	63.95	69.31	72.20
1300	78.20	96.53	93.17
1400	93.83	99.00	99.23
1500	97.81	99.67	93.75

Table 3: Crystalline phases contents of different sintered samples at 1300-1500 ^{0}C.

Temperature (^{o}C)	Y-TZP		Y-TZP10				Y-TZP20			
	t- ZrO_2 content (vol%)	m- ZrO_2 content (vol%)	t- ZrO_2 content (vol%)	m- ZrO_2 content (vol%)	$Ca_2P_2O_7$ content (vol%)	$ZrSiO_4$ content (vol%)	t- ZrO_2 content (vol%)	m- ZrO_2 content (vol%)	$Ca_2P_2O_7$ content (vol%)	$ZrSiO_4$ content (vol%)
1300	99.8	0.2	90.7	1.0	2.8	5.5	78.2	2.5	6.8	12.5
1400	99.0	1.0	88.8	2.8	3.0	5.4	70.6	10.0	6.7	12.7
1500	97.8	2.2	83.7	7.8	2.9	5.6	59.5	21.0	6.9	12.6

a

Shrinkage (%)

Temperature (^{0}C)

b

Relative density

5 $^{\circ}$C/min
10 $^{\circ}$C/min
15 $^{\circ}$C/min

Temperature (^{0} C)

c

dρ/dT (^{0}C^{-1})

Temperature (^{0} C)

Y-TZP

A

Y-TZP10

B

Y-TZP20

C