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Original

In Vitro Efficacy of $CaCO_3$ Content in $CaTiO_3$– $CaCO_3$ Composites for Bone Growth

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Abstract: The effect of $CaTiO_3$ compounded with different amounts of $CaCO_3$ on osteoblastic KUSA/A1 cells was evaluated. $CaTiO_3$-$CaCO_3$ composites were obtained by alkoxide method, a simple, low-cost and reproducible technique used for large-scale production of material. The content of $CaCO_3$ in our samples was controlled by varying the sintering time of the overall process. Composite morphology was assessed by scanning electron microscopy (SEM) showing particles with sizes ranging from100 to 500 nm. The presence of $CaCO_3$ was revealed by XRD and thermogravimetric analyses, which suggested that samples treated at 650ºC for 30 min contained higher amounts of $CaCO_3$ than samples treated for 2 and 10 h. Additionally, in vitro studies demonstrated that $CaTiO_3$–$CaCO_3$ composites sintered for 30 min induced augmented cell proliferation and mineralization in comparison to composites sintered for longer periods of time. Hence, our findings clearly suggest that the amount of $CaCO_3$ within $CaTiO_3$-$CaCO_3$ composites exerts a critical effect on osteoblastic cells response. Enhanced bone regeneration could be achieved by increasing the content of $CaCO_3$ within the composites, thus establishing $CaTiO_3$-$CaCO_3$ as a promising material for bone augmentation procedures in dental field.

Key words: Bone regeneration, Calcium carbonate, Mesenchymal stromal cells, Powders

Introduction

Calcium phosphates are at present the most biocompatible synthetic bone substitute available. However, in order to enhance their biological response, such substitutes compounded with osteoconductive and osteoinductive materials are still under analysis[1,2)]. Hence, studying new materials and their biological effects opens the possibility of new composites suitable for different applications in dental restoration field. Calcium titanate ($CaTiO_3$) has gained extensive attention in many biomaterial-related works as a promising bioactive ceramic, primarily due to its higher bone tissue biocompatibility in comparison to pure titanium (Ti) and its alloys, and given its positive surface charge which resembles electrical properties of natural bone[2)]. Up to date, several reports proved both, *in vitro* and *in vivo*, an augmented and stable biochemical bonding between $CaTiO_3$ surfaces and bone tissue in addition to an accelerated osseointegration to $CaTiO_3$-coated implants[3-6)]. Furthermore, it was shown that aforementioned bone bonding to $CaTiO_3$ might be increased either through micro- and nanostructuration of $CaTiO_3$ or by combining this material with carbon in different forms[7,8)]. Carbon-containing materials are nowadays used in multiple biomedical applications given their desirable biological response, as reported by Yamamoto & Fukuda. In their work, it was demonstrated a higher biocompatibility of carbon-film-coated Ti implants compared with bare Ti implants[9)]. Carbon coatings particularly intended for orthopedic implants have been applied either as carbon allotropes (e.g. multiwalled carbon nanotubes, diamond-like carbon, graphite-like carbon and amorphous carbon) or carbon compounds like e.g. carbon-doped apatites and calcium carbonate ($CaCO_3$)[10-16)]. Titanium surfaces coated with $CaCO_3$ particularly have shown to induce attachment, proliferation and differentiation of osteoblastic cells[17)]. In addition, Ohgushi et al., proved a comparable behavior between $CaCO_3$ and bioactive hydroxyapatite when implanted with rat marrow cells into subcutaneous sites of syngeneic rats[18)]. Regarding clinical applications, coralline $CaCO_3$ particles have been already used as graft material in human periodontal osseous defects showing a favorable osteconductivity over long periods of time[19,20)]. Then, $CaCO_3$ is widely recognized as a potential bone substitute material[21)].

Previous studies of our group compared osteoblastic cell response against $CaTiO_3$ compounded with amorphous carbon ($CaTiO_3$-aC), $CaTiO_3$-$CaCO_3$ composites, hydroxyapatite and commercial $CaTiO_3$. Results revealed that the presence of $CaCO_3$ in $CaTiO_3$ increases proliferation, differentiation and mineralization of bone marrow stromal cells in comparison to commercial $CaTiO_3$ and hydroxyapatite. Our results thus proved $CaTiO_3$-$CaCO_3$ composites as promising compounds for bone induction in medical and dental fields[22)]. Hence, the aim of this work is to evaluate the effect of different amounts of $CaCO_3$ combined with $CaTiO_3$ on the osteoblastic response of KUSA/A1 cells.

In order to maximize the advantages of $CaTiO_3$-$CaCO_3$ composites, here we synthesize the aforementioned compounds through an alkoxide method. Up to date, bioactive compounds like $CaTiO_3$ and $CaCO_3$ have been separately formed by methods such as plasma spraying, sputtering, sol-gel or complex hydrothermal techniques which either require expensive equipment, strict control of precursor hydrolysis or multiple processing steps[3)]. In this work, the alkoxide method is followed by sintering. The former technique aims to simultaneously form $CaTiO_3$ and $CaCO_3$, whereas the latter provides optimal mechanical properties for hard tissue environments. In addition, different sintering times are here used to regulate $CaCO_3$ formation within $CaTiO_3$ bulk. To the best of author's knowledge, there is no report about the effect of $CaCO_3$

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concentration in $CaTiO_3$- $CaCO_3$ compounds on cell response. Therefore, in this paper we also focus our assays on the biological effect of $CaCO_3$ on bone marrow stromal cells.

Materials and Methods

Preparation of $CaTiO_3$–$CaCO_3$ through alkoxide method

The protocol used for material preparation was previously described by Rodriguez *et al*.[22]. Briefly, calcium nitrate (Sigma-Aldrich Co., Tokyo, Japan) was dissolved in 2-isopropanol (Sigma-Aldrich Co., Tokyo, Japan) followed by addition of titanium isopropoxide (Wako Inc, Osaka, Japan). The solution was placed under a draft at room temperature for 1 week until it reached paste consistency. To transform the paste into powder, the resulting material was dried overnight at 110°C and then divided into four groups. Three of them subsequently underwent heat treatments at 650ºC for 30 min, 2 and 10 h, whereas the fourth group was subjected solely to drying treatment at 110°C. It is worth mentioning that 650°C was chosen as the sintering temperature given that it is the minimal temperature at which a crystalline structure could be identified in $CaTiO_3$-$CaCO_3$ compounds as it was determined during preliminary studies.

Material characterization

Crystal structure of samples was characterized by powder X-ray diffraction by a XRD, Rigaku RINT2100/PC (Rigaku Co., Tokyo, Japan) set at 50 mA, 200kV and using a monochromated CuKα radiation. Scanning electron microscope images were obtained on a Topcon DS-720 scanning microscope (Topcon Co., Tokyo, Japan). Thermogravimetric (TGA) analyses were carried out on a Instruments Thermoanalyzer TG-DTA-TWIN Rigaku (Rigaku Co., Tokyo, Japan). The measurements were performed under static air conditions, with a heating rate of 5ºC/min at temperatures ranging from 20 to 800°C.

Cell culture method

KUSA/ A1 cells are a marrow stromal cell line which possess osteoblastic properties in vivo. Such immortalized cells were obtained from primary bone marrow culture of a female C3H/He mouse by frequent subculture for more than a year. In particular, KUSA/A1 cells used in this work were courtesy of Dr. Umezawa A. from Keio University, Tokyo, Japan. The cells were cultured in minimum essential medium alpha medium (α-MEM, Gibco Brl Co., Ltd Life Technologies Inc., Carlsbad, CA, USA) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA) and 1% antibiotic-antimycotic agent (Life Technologies, Thermo Fisher Scientific Inc., Yokohama, Japan).

KUSA/A1 cells were cultured either with test material at a concentration of 10 mg/20 mL of medium or in the absence of material. 24-multiwell plates were used for comparing cell proliferation and differentiation features whereas 3 cm Petri dishes were utilized for mineral detection at a seed density of 7500 cells/cm^2. Cells were incubated at 37ºC in humid air with 5% CO_2.

Cell proliferation by MTS assay

The MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay was performed to assess cell proliferation using CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega Co., WI, USA). This assay measures the conversion of a methyl tetrazol sulfate (MTS) into aqueous formazan product. The cells were incubated for 1, 4, 7 and 10 d in a 5% CO_2 atmosphere at 37ºC. The medium was discarded by aspiration, and then the cells were incubated in 500 µl of the α-MEM and 100 µl of CellTiTer 96® Aqueous One Solution Reagent for 1 h in a 5% CO_2 atmosphere at 37ºC. At the end of the incubation, 120 µl of the resulting supernatant was removed to a 96 well plate to measure the absorbance at 492 nm. The absorbance of the MTS reagent incubated without cells was used as the blank value.

Determination of the Alkaline Phosphatase Activity

The measurement of alkaline phosphatase (ALP) activity was performed by p-Nitrophenyl Phosphate Substrate method (Wako Inc., Osaka, Japan), according to manufacturer directions. This assay was normalized to the total protein content (Protein assay, BIO-RAD Inc., Tokyo, Japan) of the samples. Cell cultures were examined at 1, 4, 7 and 10 d.

Mineralization assay by alizarin red staining

Cells were washed once with phosphate buffered saline (PBS) provided from Takara Bio (Shiga, Japan) and fixed with 95% ethanol at 37ºC for 15 min. The fixed cells were then washed with distilled water and subsequently stained with 1% alizarin red S (Kayayama chemical industries Co. Ltd, Osaka, Japan) solution for 5 min. The remaining dye was washed out 3 times with distilled water. The stained samples were visualized by phase microscopy using an inverted microscope.

Statistical analysis

Results are expressed as mean±standard deviation from a set of 6 values. Variance analysis in all cases was done through Levene test with a significance level of 0.05. For means comparison, One-Way ANOVA in combination with post hoc Tukey HSD test were applied. P-values of <0.05 were considered statistically significant.

Results

Material Characterization

Scanning electron microscopy

$CaTiO_3$-$CaCO_3$ powders were observed by SEM with a magnification of 20000X. SEM images indicated no marked difference between the three sintered groups of samples. As it can be seen in Fig. 1, powders were comprised of particles with diameters ranging from 100 to 500 nm approximately. Furthermore, as shown below, particles presented irregular edges and shapes in addition to a tendency to form clusters.

X-ray diffraction spectra

Structural study of the particles was assessed by XRD analysis. Fig. 2 shows XRD diffraction pattern of sintered samples at 650°C for 30 min, 2 and 10 h in addition to only-dried samples. As it can be seen in the diffractogram, all $CaTiO_3$-$CaCO_3$ powders sintered at 650°C showed peaks corresponding to perovskite-type $CaTiO_3$ at 32.9°, 47° and 59.2° (JCPDS card No. 08-0092). For a sintering time of 10 h, sharper peaks were detected, which indicate a higher crystallinity compared to the other samples. $CaCO_3$ peak at 29.47° is clearly observed for samples sintered at 30 min and 2 h, nonetheless samples sintered for 10 h showed no peak at this angle.

Thermogravimetric analysis

Decomposition process was studied by TGA and corresponding profiles are shown in Fig. 3. Only samples sintered for 30 min showed several inflexions along the curve whereas samples sintered for 2 and 10 h contrastingly showed single step decomposition spanned over a temperature range 0-800˚C. Furthermore, all of them have a maximum weight loss around 600˚C which corresponds to a typical thermal decomposition of calcium carbonate, arising from the decomposition of $CaCO_3$ to CaO. Additionally, it can be clearly seen that the sample sintered for 30 min showed the higher weight loss of carbon from 95.4%

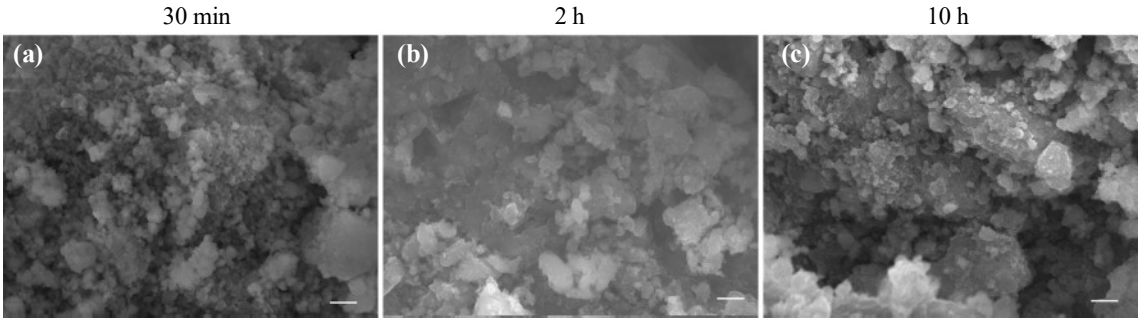

Figure 1. SEM images of $CaTiO_3$-$CaCO_3$ sintered at 650°C for (a) 30 min, (b) 2 h and (c) 10 h. Particle size ranges from 100 to 500 nm. Particles have irregular shapes and are prone to cluster formations. Scale bar=200 nm

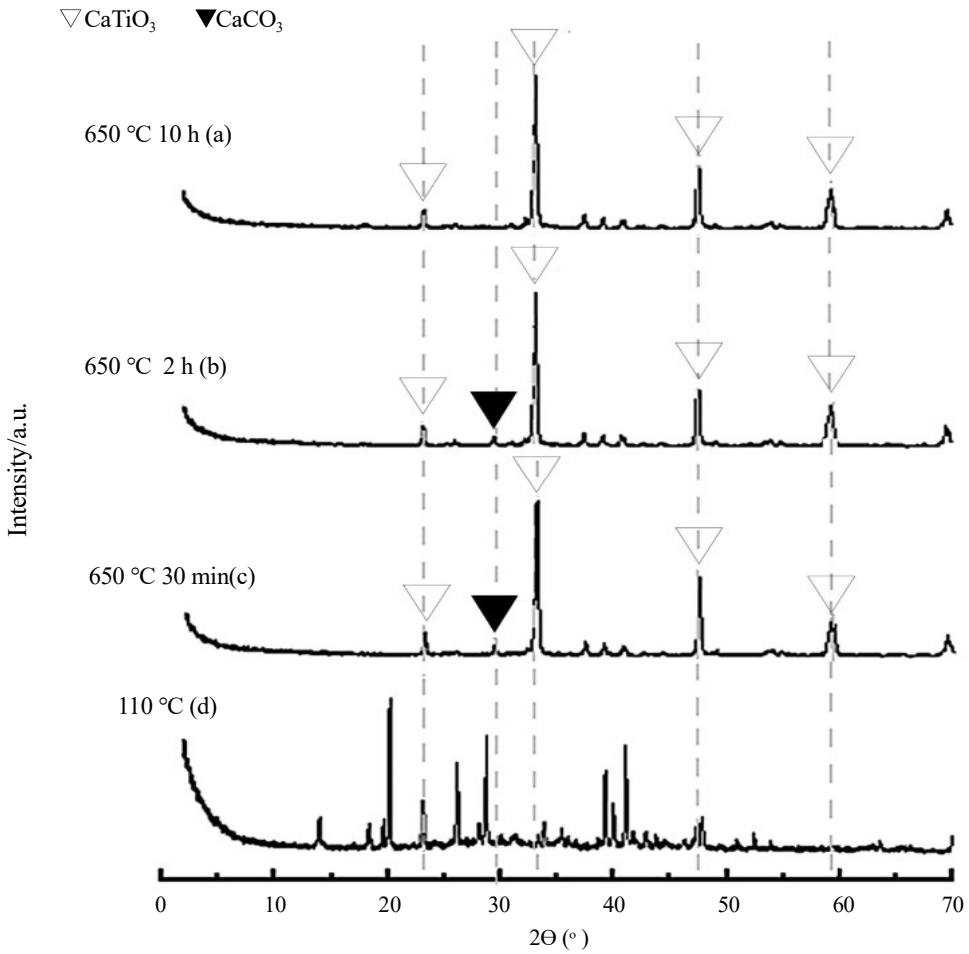

Figure 2. XRD pattern of $CaTiO_3$-$CaCO_3$ powders sintered at two different temperatures for different periods of time (a) 650°C-10h; (b) 650°C-2 h; (c) 650°C-30 min; (d) 110°C. $CaCO_3$ peaks at 29.47° are marked with black arrows. As it is observed, such a peak is not detected at spectra from 650°C-10 h sintering compounds. $CaTiO_3$ peaks at 32.9º, 47º, and 59.2º are showed with a white arrow.

to 92.5%, around 600°C, which is directly related to the content of calcium carbonate. Contrastingly, samples sintered for 2 and 10 h showed a lower amount of carbon losses against 30 min sintered samples. The final weight loss of $CaCO_3$ for samples sintered at 30 min, 2 h and 10 h correspond to 2.9%, 0.72% and 0.48% respectively. No particular weight loss was observed for $CaTiO_3$ within this temperature range.

Moreover, the first inflexion at 100˚C corresponds to a drying stage at which the weight loss is due to evaporation of water content within the powder. For the sample sintered for 30 min, water weight loss corresponds to 1.71%; this behavior was slightly seen for samples sintered for 2 and 10 h. In addition, the presence of an inflexion in the plot at temperatures between 450-550ºC, can be correlated with the loss of carbon monoxide produced during the assay.

In vitro assays

Cell proliferation

A protocol to determine the number of viable cells in culture based on the measurement of MTS activity was performed. Measurements

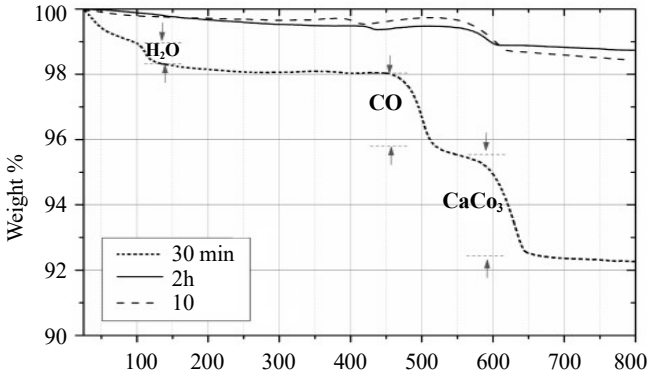

Figure 3. Thermo gravimetrical analysis of samples sintered at 30 min, 2 h and 10 h. The first inflexion at 100ºC corresponds to water weight loss due to drying. For temperatures between 450-550ºC the weight loss correlates with loss of CO produced during the assay. $CaCO_3$ decomposition into CaO occurs around 600˚C showing a weight loss of 2.9%, 0.72% and 0.48% for samples sintered at 30 min, 2 h and 10 h respectively. No weight loss was observed for $CaTiO_3$ at this temperature range.

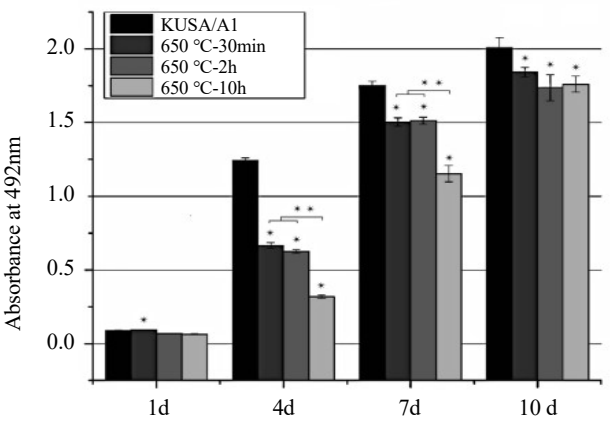

Figure 4. MTS activity of KUSA/A1 cells cultured in the presence of powder sintered for 30 min, 2 and 10 h for 1, 4, 7 and 10 d. A control group of cells in absence of the material was also cultured. $CaTiO_3$–$CaCO_3$ sintered at 650ºC for 30 min and for 2 h demonstrate a significant difference and increased proliferation compared to powder sintered during 10 h for day 4 and 7. Data represents mean ± SE, n=4. *Statistical significance against cell proliferation on control sample for the same period of culture, **statistical significance between samples for the same period of culture, ($p<0.05$).

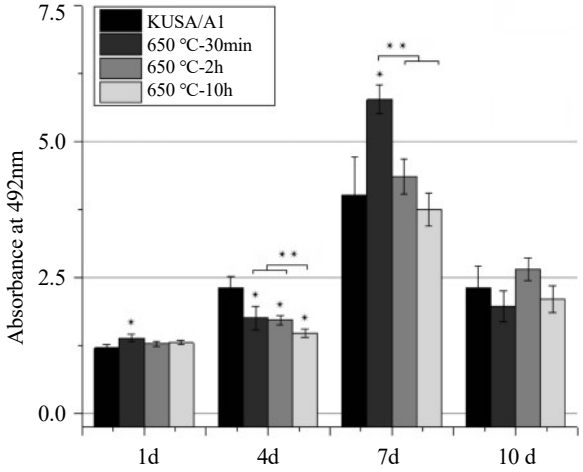

Figure 5. ALP activity of KUSA/A1 cell cultured in the presence of powder sintered for 30 min, 2 and 10 h for 1, 4, 7 and 10 d. A control group of cells in absence of the material was also cultured. By day 7 a maximum ALP expression was seen at $CaTiO_3$–$CaCO_3$ sample sintered at 650ºC for 30 min in comparison to the other powders. *Statistical significance against cell ALP activity on control sample for the same period of culture, **statistical significance between samples for the same period of culture, ($p<0.05$).

were made at 1, 4, 7 and 10 culture days of KUSA/A1 cells in contact with the different powder groups (Fig. 4). Results indicated that cell viability increased in all groups after 4 d. Interestingly, cells cultured with $CaTiO_3$–$CaCO_3$ sintered at 650ºC for 30 min and for 2 h demonstrated a significant difference and increased proliferation compared to powder sintered during 10 h at days 4 and 7, $p<0.05$.

Alkaline phosphatase activity

Osteoblastic differentiation was analyzed by measuring ALP activity. Fig. 5 summarizes the ALP activity of KUSA/A1 cells cultured on different powder samples during 1, 4, 7 and 10 d. ALP activities reached a maximum expression on day 7 and then decreased on day 10 in all samples. $CaTiO_3$–$CaCO_3$ samples sintered at 650ºC for 30 min demonstrated a significant increase of ALP activity levels at day 7 compared to the other powders, $p<0.05$.

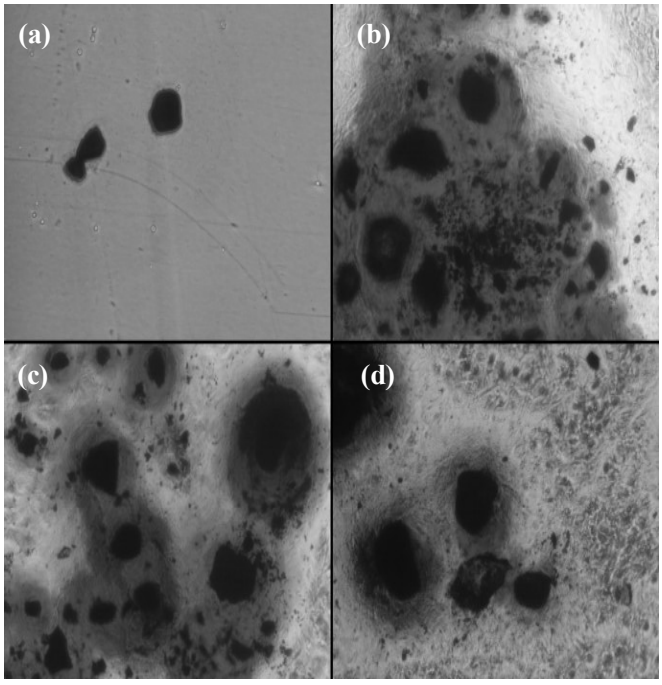

Figure 6. Alizarin red staining for KUSA/A1 cells with $CaTiO_3$- $CaCO_3$. Material alone as negative control (a), cells cultured onto $CaTiO_3$- $CaCO_3$ sintered at 650ºC for 30 min (b), 2 h (c) and 10 h (d). Powders cultured with KUSA/A1 cells induced mineralized matrix around each particle. $CaTiO_3$- $CaCO_3$ sintered for 30 min showed larger area of calcification compared to the other groups.

Detection of mineralization

Alizarin red staining was used to examine the mineralization potential of KUSA/A1 cells on $CaTiO_3$-$CaCO_3$ powders with different sintering times. Fig. 6 shows that $CaTiO_3$-$CaCO_3$ alone did not reveal any sign of mineralization. In contrast, all powders cultured with KUSA/A1 cells were capable of inducing mineralized matrix around each particle. Moreover, the particles sintered during 30 min demonstrated larger area

of calcification than other groups.

Discussion

Alkoxide method is a simple, low-cost and reproducible technique that allows a simultaneous production of significant amounts of $CaTiO_3$ compounded with $CaCO_3$. In this work we showed that it is possible to control the quantity of $CaCO_3$ within the material by varying the sintering time in the overall process (Figs. 2 and 3).

It is well known that carbon-containing materials improve biological response as we showed during previous studies of our group, where we demonstrated that calcium carbonate in $CaTiO_3$–$CaCO_3$ has a good osteoblastic response[22]. Hence, here we examined the effect of different amounts of $CaCO_3$ combined with $CaTiO_3$ on cell proliferation and osteoblastic response. Three different heat treatments were studied: 650°C for 30 min, 2 h and 10 h; all of them produced particle sizes ranging from 100 to 500 nm, approximately (Fig. 1). XRD profiles showed a pure phase microstructure of perovskite-type $CaTiO_3$ at all samples in addition to the lack of undesired compounds due to composite processing (Fig. 2). An increased crystallinity for $CaTiO_3$ samples sintered at 650ºC for 10 h can be seen given the sharper peaks observed in the diffractogram compared to the other samples. This behavior was also seen by Bandyopadhyay et al. when preparing nanocrystalline $CaTiO_3$ powders doped with Fe_2O_3[23]. A $CaCO_3$ peak at 29.47° was clearly observed for 30 min and 2 h sintered pattern samples, but the same disappeared for samples heat treated for 10 h. This might be caused by decomposition of $CaCO_3$ into CaO and CO_2, which is also reflected in TGA results as its decomposition is characteristic around 600ºC. Also, $CaCO_3$ presence was revealed by XRD and TGA analyses, which showed that powders treated at 650ºC for 30 min had higher contents of carbon than powders treated at the same temperature for 2 and 10 h (Figs. 2 and 3). Moreover, further material characterization by FT-IR analysis performed in previous study elucidated as well the presence of $CaCO_3$ within the composites[24]. FT-IR spectra presented bands at 1430 and 870 cm-1 which correspond to asymmetric stretching and asymmetric deformation of carbonate group (CO_3), respectively. These vibration frequencies correspond to one of the polymorphs of $CaCO_3$ named calcite[25]. Also, it was observed that band intensity increases with decreasing sintering times, achieving a maximum for 30 min of sintering[24]; these results are in accordance with current findings. Three different crystalline and one amorphous phase of anhydrous calcium carbonate can be found in nature, these are: calcite, aragonite and vaterite. Based on previous studies, herein, we chose an annealing temperature of 650ºC in order to produce the calcite-type $CaCO_3$ which is considered the most stable form at ordinary temperatures and pressure[26]. Moreover, it is known that $CaCO_3$, either in the form of aragonite or calcite, is biocompatible, osteoconductive and can promote in vitro cell proliferation and differentiation of mouse osteoblast precursor cells[27] and of human bone marrow cells[28]. Hence XRD, TGA and former FT-IR results confirm the presence of $CaCO_3$ in the form of calcite and an indirect relation between sintering time and $CaCO_3$ quantity.

In order to elucidate the *in vitro* cell response by $CaTiO_3$ with different amounts of $CaCO_3$, we studied its influence on cell proliferation, osteoblastic differentiation and mineralization. Our results demonstrated that cells cultured with $CaTiO_3$–$CaCO_3$ sintered at 650ºC for 30 min and 2 h showed an increased cell proliferation with significant difference compared to the powder sintered during 10 h at culture days 4 and 7 (Fig. 4).

Osteoblast-like response of KUSA/A1 cells was analyzed in terms of in vitro ALP activity and mineralization assessment. KUSA/A1 cells, a bone marrow stromal cell line, are capable of maintaining an immature stage when cultured in α-MEM even at high cell proliferation rates. Such a cell line solely induces mineralized nodules when cultured in specific osteogenic medium[29,30]. Thus, to determine $CaTiO_3$–$CaCO_3$ effect on KUSA/A1 response, the cells were cultured in α-MEM together with the different compounds synthesized. In previous work we observed that the maximum ALP activity increased at day 7 in $CaTiO_3$–$CaCO_3$ sintered at 650ºC for 2 h compared to $CaTiO_3$–aC, $CaTiO_3$ and HA indicating its beneficial effect for osteoblastic differentiation[22]. Moreover, the expression of osteogenic genes was higher in poly(ε-caprolactone)-calcium titanate systems in comparison to poly(ε-caprolactone)-strontium titanate and poly(ε-caprolactone)-barium titanate composites when using perovskite ceramic nanoparticles as fillers in polymer scaffolds, as MCGEE-RUSSELL reported[31]. Our results showed that ALP activities reached a maximum expression at day 7 followed by a decrease on day 10 in all samples, where samples sintered at 650ºC for 30 min, interestingly, showed the highest increment compared to the other powders (Fig. 5). Alizarin Red S staining is normally used to determine the presence of calcium deposition on matrix mineralization induced by osteoblasts in cell culture, either bone or other calcified structure[32]. In this work, mineralization assessment through Alizarin Red S staining clearly revealed mineral deposits produced by KUSA/A1 cells when cultured with $CaTiO_3$–$CaCO_3$ compounds suggesting an osteogenic response. Furthermore, particles heat treated for 30 min demonstrated larger areas of calcification compared to samples treated for 2 and 10 h, proving that higher contents of $CaCO_3$ induce extended mineralized areas (Fig. 6). Such effect was observed as well when KUSA/A1 cells were cultured in usual α-medium and in contact with $CaTiO_3$–aC and HA during previous studies[22]. The increased mineralized areas observed in cultures with higher amounts of $CaCO_3$ might be caused by increased amounts of Ca^{2+} released to culture medium. *In vitro* degradation of different $CaCO_3$ samples compared to β-TCP and HA after immersion in Tris-HCl solution was reported in literature[21]. Within this degradation model, only material dissolution followed by particle disintegration is involved. HE *et al*., showed that $CaCO_3$ sintered at a low temperature (650 °C) is not robust enough and high degradation might be seen[21]. Moreover, compounds with higher contents of $CaCO_3$ are degraded at a higher rate[33,34]. Hence, we consider that $CaTiO_3$-$CaCO_3$ heat treated for 30 min and therefore with higher $CaCO_3$ content might induce rapid release of Ca^{2+} to the environment resulting in larger areas of mineralized matrix around the cells.

From the present investigation, it can be therefore concluded that $CaTiO_3$ and $CaCO_3$ biocomposites were successfully fabricated using an alkoxide method which allowed controlling the amount of $CaCO_3$ by applying different sintering times. Also, an inverse relationship between the $CaCO_3$ quantities within $CaTiO_3$-$CaCO_3$ composites and sintering time was revealed, for longer heating times a decrease in the amount of C was observed. $CaTiO_3$–$CaCO_3$ material sintered for 30 min plays a more dominant effect on cell proliferation, osteoblastic differentiation and mineralization of bone marrow stromal cells than material sintered at 2 and 10 h. Our findings indicate that $CaTiO_3$–$CaCO_3$ plays a dominant and favorable effect on cell response when sintered for 30 min and could be used in further studies as an additive in bone augmentation materials.

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Conflict of Interest

The authors declared that they have no conflict of interest.

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