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Chitosan/poly(DL,lactide-co-glycolide) scaffolds for tissue engineering

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Abstract Chitosan/poly(DL-lactide-co-glycolide) (Ch/DL PLG) composite scaffolds were fabricated by freeze-drying lyophilization, and were evaluated and compared for use as a bone regeneration scaffold through measurements of the compression mechanical properties of the porous scaffolds. Also, In vitro cell culture of Sprague–Dawley rat's osteoblasts were used to evaluate the phenotype expression of cells in the scaffolds, characterizing the cellular adhesion, proliferation and alkaline phosphatase activity. The gene expression of osteocalcin, sialoprotein, alkaline phosphatase, Type I collagen and TGFβ1 were confirmed in the samples; moreover, it was confirmed, the mineralization by IR spectra and EDS analysis. Our results thus show that Ch/DL PLG scaffolds are suitable for biological applications.

1 Introduction

Significant efforts have been made in the field of tissue engineering to develop new tissue substitutes of polymer scaffolds that perform the structural and biochemical functions of extracellular matrix (ECM). In essence, the scaffolds are seeded with cells according to tissue engineering principles, being biocompatible, nontoxic, having with suitable mechanical properties, being biodegradable and adequate for the diffusion of gases and nutrients in order to support proliferation [1–5]. In order to obtain such structure, several preparation methods to get porous scaffolds have been used, such as solvent casting, porous woven structures, thermal-induced phase separation, freeze-drying and gas foaming [6].

Biodegradable synthetic polymers such as poly(glycolic acid), poly(lactic acid), poly (DL-lactic-co-glycolic acid), or naturally derived polymers such as collagen, glycosaminoglycan and hyaluronic acid have been used to fabricate scaffolds [7–9] in combination with other polymers to improve its properties [7, 10].

Chitosan is a linear polysaccharide that consists of 2 amino-2-deoxy-β-D-glucopyranose and 2-acetamido-

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2-deoxy-β-D-glucopyranose. Chitosan has unique properties such as bioactivity, biocompatibility and biodegradable [7]. It is a deacetylated product of chitin, which is obtained from exoskeletons of insects, shells of crustaceans and fungal cell walls [11, 12]. It has been used in drug delivery vehicles, orthopedic materials, surgical devices, wound healing, wound dressing, packing, and scaffolds for tissue engineering [5, 13]. Chitosan-based blend have been studied as a potential material in regenerating bone tissue, but have showed inferior biomechanical properties [14, 15].

The poly(lactide-co-glycolide) (PLGA) is one of the most frequently studied class of biodegradable polymers because it does not require surgical removal and can be easily processed in different shapes [16, 17]. Furthermore, it is a biodegradable polymer with adjustable degradation rate and good biocompatibility. PLGA has been used as a suture material, drug carrier and orthopedic device [5]. Also, it has been used as a scaffold for bone tissue engineering, due to its physical properties, such as better mechanical properties than chitosan [14]. According of above mentioned characteristics, it can be seen that poly(DL-lactide-co-glycolide) and chitosan have complementary abilities. Therefore, it is reasonable to expect that their individual deficiencies would be improved with a blend of these materials. Previously, several efforts have been devoted to produce an homogenous mixture of poly(DL,lactide) or poly (L,lactide) with chitosan by solution casting and solvent-extracting [3, 18, 19], but not using poly (DL-lactide-co-glycolide) (DL PLG).

The interactions between cells and these biomaterials should be evaluated in primary culture systems to analyze how the cells responds to the composite, due that to the in vitro biocompatibility in these materials has been mainly evaluated in immortalized cells lines [20] or other cell types, such as chondrocytes [3].

In order to evaluate the potential applications in bone engineering, a chitosan/poly (DL-lactide-co-glycolide) (Ch/DL PLG) composite was produced as a scaffold using phase separation. In this study we investigated the behavior of primary osteoblast cultured in the presence of chitosan and poly(DL-lactide-co-glycolide). Then, biomineralization, alkaline phosphatase (ALP) activity, cell viability and gene expression of osteoblasts in the scaffolds were evaluated.

2 Materials and methods

2.1 Materials

To prepare the Ch/DL PLG composites, a sample of chitosan was purchased from Carbomer, Inc (United States). 75:25 Poly (DL-lactide-co-glycolide) (DL PLG) with an inherent viscosity range of 0.55–0.75 dK/g in $CHCL_3$ was purchased from Lactel (United States). Ethanol was provided by CTR Scientific (Mexico). Chloroform (CTS scientific, Mexico) and glacial acetic acid (Mallinckrodt, United States) were used as solvents. PBS was prepared in our laboratory.

2.2 Preparation of composites

Chitosan/DL PLG composites with different compositions were prepared and lyophilized in our laboratory using a previously reported method [21]. The lyophilized composite was neutralized with a treatment of NaOH–CH_3CH_2OH. Finally all neutralized scaffolds were pre-conditioned and sterilized with absolute ethanol for 10 min followed by 4 h of UV light exposition on each side of the scaffold. Then each scaffold was degasified with Alpha Mem with 10 % Fetal Bovine Serum in an incubator at 37 °C overnight before it was used for culture with cells.

2.3 Mechanical properties

RSA III (Rheometrics Analyzes System) TA was used in static mode at a frequency of 6.2832 rad/s and at 37 °C for all samples. Disks of approximately 10 mm in thickness and 25 mm in diameter were used in all the experiments. At least three samples of each composition were measured and the results were averaged. Samples were mounted using a compression clamp.

2.4 Cell harvesting and culture

About 14-day old rats Sprague–Dawley were used for the isolation of osteoblasts from calvarian. For the isolation of osteoblasts from rat calvaria, we used the method of Anagnostou [22]. The calvaria tissue were dissected from 14 days old Sprague–Dawley and prepared by enzymatic digestion using collagenase (type I) in phosphate buffered saline (PBS) at 37 °C. The cells were dissociated from bone fragments by repeated pipetting (mechanical effect). National guidelines for the care and use of laboratory animals were observed.

The scaffolds (1 cm^2 approximately) were sterilized in an ethanol bath for 5 min and washed with sterile DI water. Then they were placed in a 24 well-culture plate and each side of the scaffolds was exposed to UV light in a laminar flow hood for 12 h. After this treatment, 300 µl of α-Mem supplemented with 10 % fetal bovine was added to each scaffold and incubated at 37 °C overnight to degas and to prepare the surface of the sample for the cell culture.

Once the previous process was finished, osteoblasts calvaria cells, were pooled into the scaffolds at a density of 200,000 cells/cm^2 and cultured in the prepared solution of α-MEM supplemented with 10 % fetal bovine, 1 %

antibiotics, 2.1 mg/ml β-glycerophosphate and 50 μg/ml ascorbic acid in an incubator at 37 °C with 5 % CO_2. The medium was replaced carefully every 48 h without disturbing the scaffolds. Cultures were finished at 3, 7, 14, 21 and 28 days.

2.5 Scanning electron microscopy (SEM) examination of cell-seeded scaffolds

After being cultured for various numbers of days, the samples with attached cells, were rinsed twice with PBS (pH 7.4), and immersed in PBS containing 3 % Glutaraldehyde for 4 h to fix the cells, followed by rinsing twice with PBS for 10 min. They were dehydrated through a series of graded ethanol solutions (from 30 to 100 %) for additional 30 min, being allowed to air dry overnight. The dry cell-seeded scaffolds were mounted on aluminum stubs. A field emission scanning electron microscopy JEOL JSM-7000F coupled with an energy-dispersive system EDS 7557 INCA Oxford Instruments (England) was used to characterize the morphology and cell proliferation.

2.6 Biochemical and differentiation analyses

The ALP activity of the osteoblasts on the scaffolds was measured. As a control osteoblasts were seeded directly on the well with the medium but without scaffold. Scaffolds with osteoblasts calvaria cells with a density of 200,000 cells/cm^2 and cultured in the prepared solution of α-MEM supplemented with 10 % fetal bovine, 1 % antibiotics, 2.1 mg/ml β-glycerophosphate and 50 μg/ml ascorbic acid in an incubator at 37 °C with 5 % CO_2 were used as a culture test. A set of control and test cultures were harvested on days 14, 21 and 28 and washed twice with PBS. The osteoblasts were lysed with 3 ml of 1 % Triton X-100 in DEPC-treated water and three freeze–thaw cycles at −70 °C. The ALP activity in the lysed cells was determined using an ALP substrate assay kit (Pierce biotechnology, Monterrey, Mexico) with p-nitrophenyl phosphate as substrate. The ALP activity was measured by spectrophotometry. The absorbance was measured at 405 nm (Microplate spectrophotometer Bench mark plus, BIO-RAD).

2.7 Cell adhesion and proliferation assay

For the adhesion assay osteoblasts were seeded onto scaffolds and then were allowed to adhere for 1 h and 4 h in the culture medium. At the end of the incubation period, the adherent cells were trypsin-detached and counted using a hemocytometer. All adhesion tests were done in triplicate. The adherence of the cells was expressed by the percentage of osteoblasts trapped in the scaffolds to the total cells seeded.

Alamar blue staining was used to measure the proliferation. The reactive was added in the sample-containing wells (final concentration 10 % in medium v/v) and the cells were incubated at 37 °C for 4 h. Absorbance was measured to produce a calibration curve, for which a number of cells (measure with a hematocytomer as direct cell counting technique) was used and seeded in a 96 well plate and incubated at 37 °C for 4 h. After the required incubation, the optical density was measured at 570 and 600 nm, as the reference wavelength in a microplate reader (Bench mark plus, BIO-RAD). The cell number corresponding to the optical density was used to plot a standard curve. This calibration curve showed an $r = 0.999$ of linear relation of the cell number with absorbance, thus showing a linear increase with cell number. Due to the correlation of number of cells and absorbance of Alamar Blue, the number of cells can be quantified spectrophotometrically without the need for counting [23]. Finally, in order to measure proliferation, the previously generated calibration curve was used with a number of cells known at 570 nm.

2.8 Quantification of gene expression by RT-PCR

Total RNA was extracted from culture cell with biomaterial incubated using TRIZOL method. The First stand cDNA synthesis was performed by reverse transcription using ImProm-II™ Reverse Transcription System Kit (Promega) and (dT)12-18 primer following the product's instruction manual.

cDNA of cells post-incubation at 0, 3, 7, 14 and 21 days, were measured by RT-PCR using specific primers. The amplifications were performed in a 25-μl reaction volume containing 100 ng of cDNA template, 12.5 μl Master mix (Promega), 1.0 μl at 20 mM of each of the forward and reverse primers (the primers used codified to Sialoprotein, type I collagen (Col I), Osteocalcin, ALP, and TGFβ1).

The cycling number of PCR was optimized to 30 cycles. The cycling protocol was 1 cycle of 94 C for 5 min; 29 cycles of 94 C for 30 s, the temperature used was depending the primer evaluated.

Primers were used to amplify GADPH as housekeeping gene. The cycling protocol was 1 cycle of 94 °C for 5 min; 29 cycles of 94 °C for 30 s, 60 for 1 min and 72 for 1 min followed by 1 cycle of 72 °C for 10 min.

Data analysis of the RT-PCR was performed by densitometry using the software EDAS-290 Kodak.

2.9 Statistical analysis

The data obtained were evaluated for statistical significance using the Student's *t* test. The results are reported as mean ± SD and the differences observed between composites results were considered significant when $P < 0.05$.

3 Results and discussion

3.1 Mechanical properties of the scaffolds

For this analysis a dynamical-mechanical instrument was used. The physical properties such as morphology and mechanical strength affect the ability of the scaffold to be used in tissue engineering. For this reason, during this study we evaluate the morphology and compression strength of the scaffolds. Figure 1 shows SEM images of the scaffolds. The composites exhibit open porous and interconnected architecture, a necessary requirement for in vitro and in vivo tissue formation [24]. Figure 2 exhibits the strain–stress deformation curves for the chitosan and the Ch/DL PLG composites and Table 1 shows the mechanical properties of the composites. The 30/70 composite exhibited the biggest module indicating that it is a more resistant material than chitosan and the 50/50 composite.

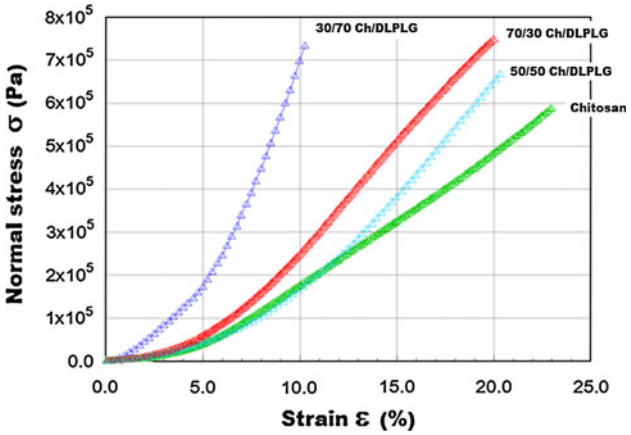

Fig. 2 Strain–stresss diagram of the Ch/DL PLG composites. The *graph* indicates that mechanical properties improves in the 30/70 composite as compared with the other composites

As DL PLG increases, the elastic module varies from 3.79×10^6 Pa to 11.4×10^6 Pa, corresponding to chitosan and 30/70 Ch/DLPLG respectively. The 30/70 composite

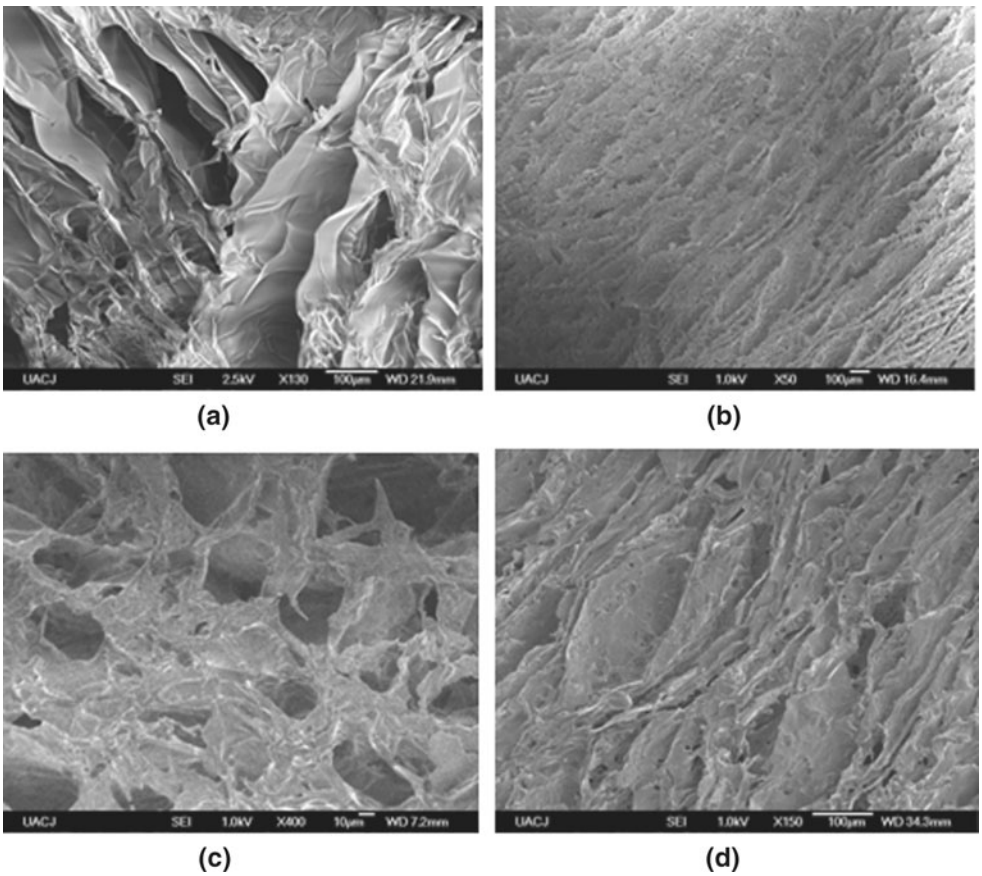

Fig. 1 SEM micrographs of **a** Chitosan, **b** 30/70, **c** 50/50 and **d** 70/30 Ch/DLPLG scaffolds before incubation

Table 1 Mechanical properties of the Ch/DLPLG composites

Composite Ch/DLPLG	Elastic module (E) (MPa)	Compression strength (Pa)	Deformation at break (%)	σ_{10} (Pa)
Chitosan	3.79	5.89×10^5	22.98	1.77×10^5
70/30	4.73	7.28×10^5	21.54	1.07×10^5
50/50	5.34	6.68×10^5	20.23	1.73×10^5
30/70	11.4	7.33×10^5	10.25	7.33×10^5

displayed a considerable increase in the elastic module when compared to the 50/50 composite and chitosan. These results exhibited improved mechanical properties for the chitosan using DL PLG for the use in tissue engineering. The mechanical properties of DL PLG used in this study were reported by the company from which the lactide was purchased. Although the existence of weak hydrogen bonding between the lactide and chitosan was expected [19], an improvement in the mechanical properties of the chitosan was observed as the DL PLG content was increased in the composite. This hydrogen bonding can be between the amino groups of chitosan and carboxiles groups of DL PLG, or between hidroxiles in the chitosan and carboxiles of DL PLG in the mix, as it has been suggested by previous research [21]. Chitosan is a semicrystalline polymer, while DL PLG is amorphous. The increase in E and σ10 could be attributed to an increase of the crystallinity degree due to the presence of both substances [19, 21].

3.2 Adherence

Adherence is an important property of a material destined to tissue engineering since it allows better proliferation, cell differentiation and influences their morphology [25]. Figure 3a shows the osteoblast adherence in scaffolds of chitosan and the composites; the adherence explains the scaffolds ability to support cell adhesion. After 1 h, the adherence showed by chitosan was more than the composites, which did not show significant difference at this time. Finally, after 4 h, significant differences between the adherences of the composites were not found, although increase an in all cases with respect to the initial was observed.

These results suggest that the incorporation of the chitosan does not improves significantly the cellular adhesion, neither that the increasing of lactides proportion affect that.

3.3 Cell viability

The cell proliferation and viability of the cells was measured using Alamar blue. The reactive changes color from blue to

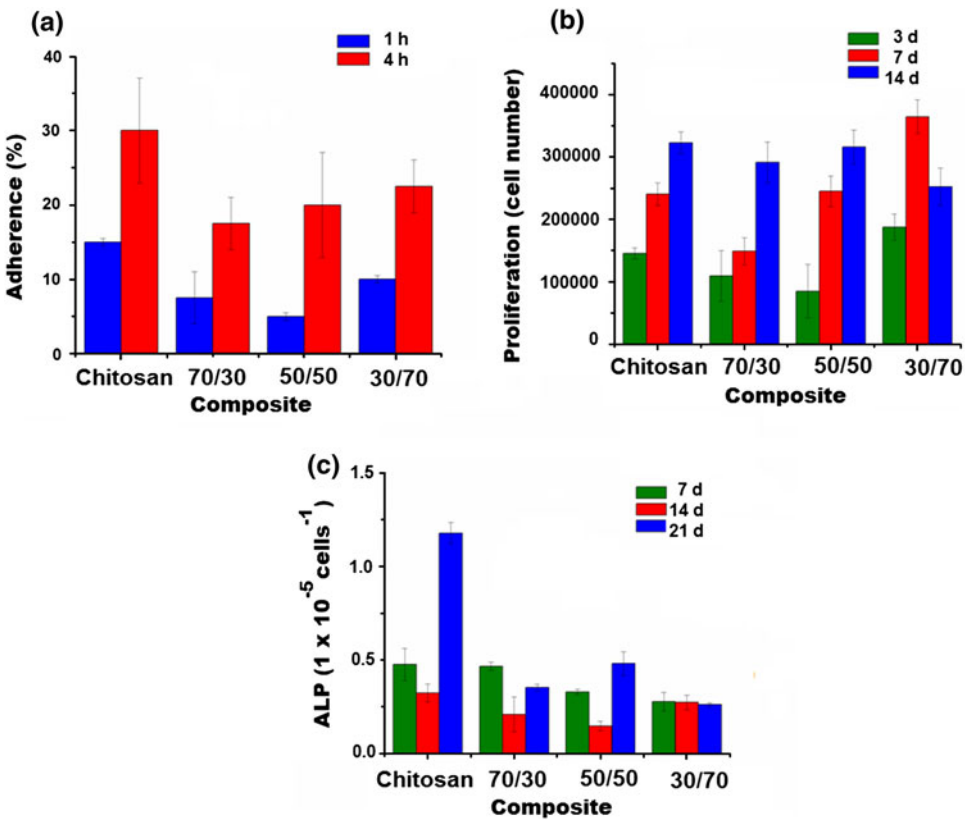

Fig. 3 **a** Osteoblasts adherence in the scaffolds at 1 and 4 h, **b** cell number at 3, 7 and 14 days in the culture plates and **c** optical density of the ALP of the cells in the scaffolds at 405 nm

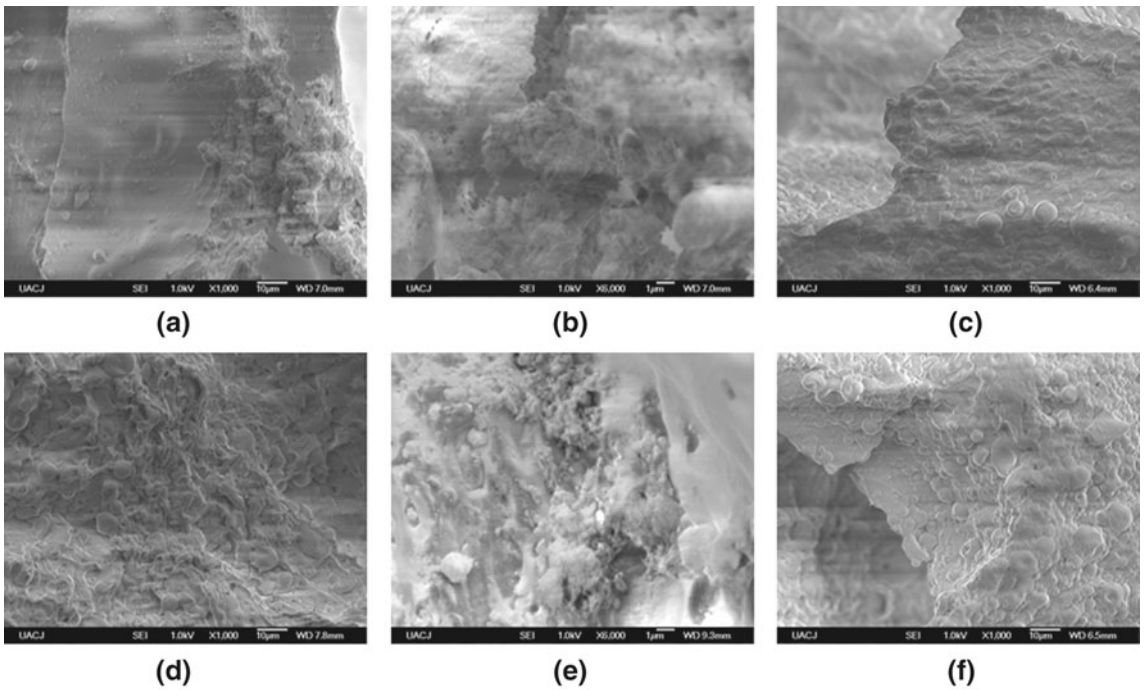

Fig. 4 SEM micrographs exhibit osteoblasts proliferation at 14 days in chitosan at **a** ×1,000 and **b** ×6,000, **c** 70/30 Ch/DL PLG at ×1,000, **d** 50/50 Ch/DL PLG at ×1,000 and **e** ×6,000, **f** 30/70 Ch/DL PLG at ×1,000. It is possible to observe that at 14 days it was visible the formation of a collagenous extracellular matrix covering the surfaces of the chitosan and 50/50 Ch/DLPLG scaffolds, while this matrix was not evident for the others composites

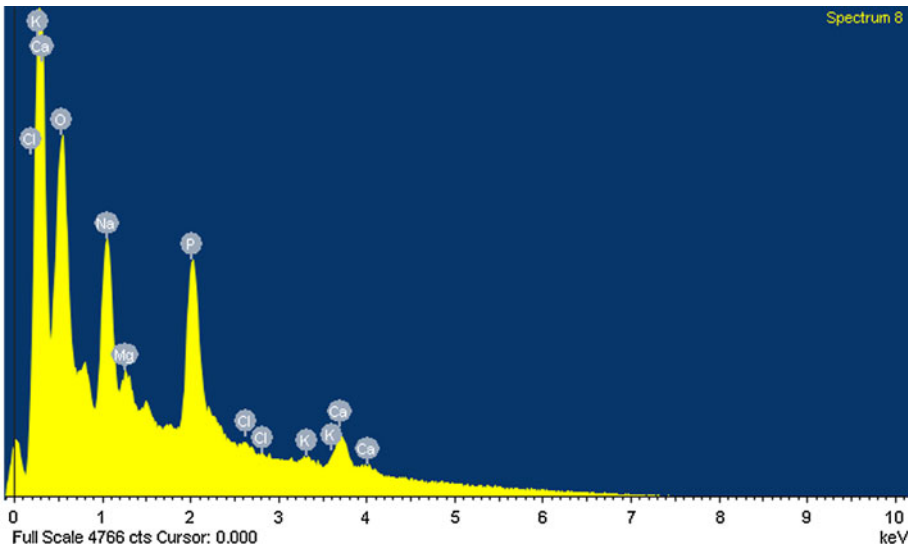

Fig. 5 EDS analysis result of the 70/30 Ch/DLPLG composite seeded with osteoblast at day 14

purple according to the metabolic activity of the cells [26]. A bar graph for behavior of the cell number of osteoblasts seeded on the different scaffolds is shown in Fig. 3b.

At day 3 the composite 30/70 Ch/DL PLG shows a significantly higher number of cells than the rest of the composite ratios. After 7 days, the tendency was similar within the same level of significance. Nevertheless, after 14 days the cell number showed by almost all the composites was similar, except for 30/70 scaffold that displayed slightly reduced proliferation. These suggest that the chitosan allows increased viability in the different combinations, although the pore size decreases as the lactide content is increased [21]. Furthermore, the lactide content is related with the acid degradation products delivered during the culture. Although no significant differences were found between day 3 and 7, the 70/30 and 50/50 composites showed a significant increased in cell number from day 3 to 14.

3.4 Biochemical and differentiation analyses

The primary culture of osteoblasts can express specific signs of phenotype such as ALP, osteocalcine and type I collagen, in the same temporal order like in vivo expression. ALP has been associated with the mineralization process and an important inference related with its function is the association between the presence of the enzyme and bone mineralization. An increased level of the ALP is a sign of the metabolic activity of the osteoblasts [27].

The bar graph in Fig. 3c illustrates the results of the ALP activity of the cells in the scaffolds. All composites showed ALP expression, indicating that the osteoblasts were able to begin the differentiation process. The behavior of ALP activity for the osteoblast is typical during the early stages of differentiation, as the increase in activity is followed by a drop, which coincides with the initiation of the mineralization process [28].

Figure 4 shows the presence of a large amount of globular mineral deposits attached to the scaffolds that was also confirmed by SEM micrographs and EDS analysis (Fig. 5). It was possible to observe the formation of a collagenous ECM covering the surfaces of the chitosan and 50/50 Ch/DL PLG scaffolds at 14 days. The presence of these matrices is important since the cells connect with each other through them as it is evident in Fig. 4b, f. Other composites do not show this matrix however, since day 7, it was possible to observe calcium deposits bonding to the cells. The FTIR spectra (Fig. 6) of the Ch/DL PLG composites seeded with osteoblasts at day 21 shows evidence of carbonate (CO_3^{2-} stretching group) at 1,458 cm^{-1} and also revealed the presence of the v_3 stretching mode of P–O bonds [P–O stretching (v_3) vibration] [29, 30].

3.5 mRNA expression

In order to examine a more immediate response to the loading matrix protein gene expression of sialoprotein, type I collagen (Col I), osteocalcin, ALP, and TGFβ1, were measured 12 h after a single bout of 2 h of loading or

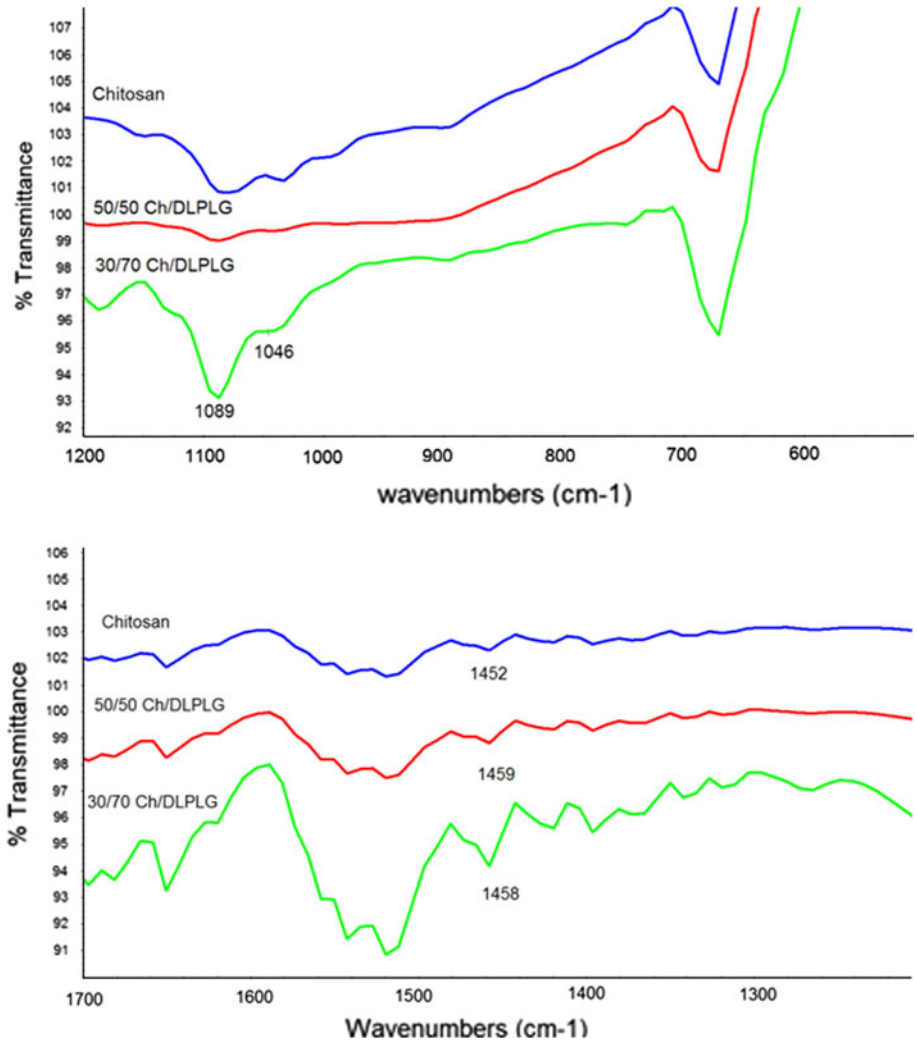

Fig. 6 FTIR of the Ch/DL PLG composites seeded with osteoblasts at day 21. The image shows evidence of carbonate (CO_3^{2-}). Also the FTIR spectra revealed the presence of the v_3 stretching mode of P–O bonds

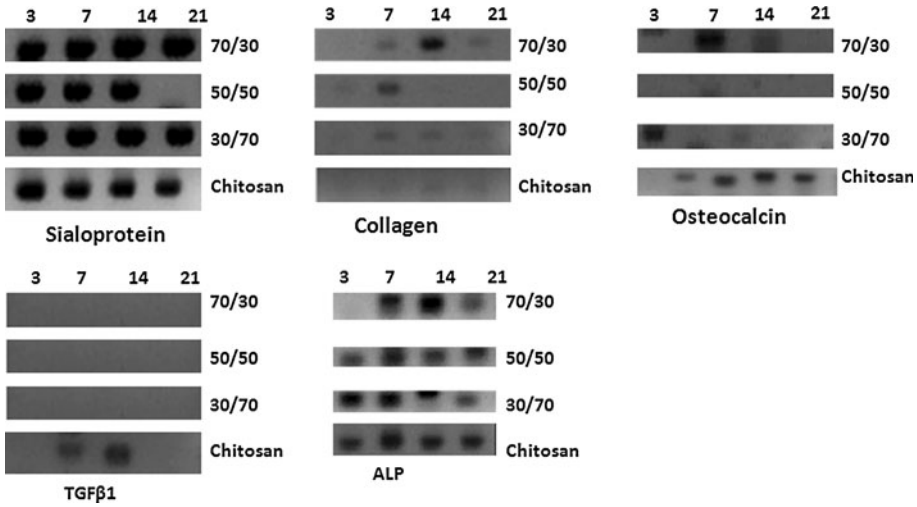

Fig. 7 Gene expression on scaffolds seeded with osteoblasts. The image shows mainly the expression of sialoprotein, osteocalcin and ALP

control treatment. In general, the expression of all markers was confirmed in all samples. Figure 7 shows that sialoprotein was expressed with all scaffolds loaded with osteoblast at 3, 7, 14 and 21 days, except by the 50/50 sample at 21 days. Type I collagen, related to the development and maintenance of osteoblast phenotype [4] and predominant in mineralized bone and osteogenic lineage [31], was expressed at days 7 and 14 on samples 50/50 and 70/30 respectively. In other samples Type I collagen's gene levels were minimal, with a slightly increased expression at day 14. On the other hand, the chitosan sample showed osteocalcin transcript expression since day 3, increasing at days 7 and 14 and decreasing at day 21. Sample 70/30 showed similar behavior. Samples 50/50 and 30/70 showed less ostecalcin expression than the other samples. This is in agreement with the behavior showed during the mineralization test, such as the biochemical analysis of ALP and its gene expression. Osteocalcin is considered a late osteoblast differentiation marker and indicates the initiation of bone matrix maturation [31, 32]. On the other hand, ALP was expressed by all loaded samples, showing increased activity at day 7 and 14. These results suggest that primary osteoblasts grown on 70/30 scaffolds promote the expression of the osteoblast phenotype better that the other compositions scaffolds and chitosan. Also, considering the ALP expression, these results provide evidence that the scaffolds promote osteoblast maturation.

4 Conclusions

The surface of DL PLG is hydrophobic, which unfavorably influences its biocompatibility during the contact with the biological environment. The use of chitosan allows the increase the hydrophilicity of the composite enhancing the penetration of culture medium and an improved adherence and proliferation of cells.

Primary osteoblasts are known to form bone tissue when implanted in vivo and during this study we determined their ability to adhere, proliferate and differentiate on the composite scaffolds. We demonstrated that the proliferation, maintaining the osteogenic phenotype, improves in the 70/30 composite as compared with the other composites, and with better mechanical properties and collagen expression than chitosan. Also, we showed that the Ch/DL PLG scaffolds are nontoxic, easily fabricated, and provide porosity with good interconnectivity for biological applications. Further studies are required to demonstrate the applicability of the composite scaffold in vivo.

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Conflict of interest declaration The authors have no conflict of interest to declare.

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