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Influence of freezing temperature and deacetylation degree on the performance of freeze-dried chitosan scaffolds towards cartilage tissue engineering

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ABSTRACT

Chitosan-based porous structures have been significantly studied across the world as potential tissue engineering scaffolds. Despite the differences in chitosan produced from squid pens or crustacean shells, with the former being more reactive and easily available with a higher degree of deacetylation (DD), most of the studies report the use of crab or shrimp chitosan as they are readily available commercial sources. The aim of this work was to highlight the great potential of chitosan produced from squid pens for biomedical application. From freeze-dried scaffolds for soft tissue engineering, we investigated the influence of the DD of chitosan and the freezing temperature during processing on their performance. Chitosan was obtained by deacetylation of β-chitin previously isolated from endoskeleton of giant squid *Dosidicus gigas* (DD 91.2%) and compared with a commercially available batch obtained from crab shells (DD 76.6%). Chitosan solutions were frozen at −80° C or −196° C and further freeze-dried to obtain 3D porous structures (scaffolds). Scaffolds prepared at −196° C have a compact structure with smaller pores, while those prepared at −80° C showed a lamellar structure with larger pores. The compressive modulus varied from 0.7 up to 8.8 MPa. Both types of scaffolds were stable on PBS, including in the presence of lysozyme, up to 4 weeks. Furthermore, the squid chitosan scaffolds processed at −80° C promoted ATDC5 chondrocyte-like cells adhesion and proliferation. The results suggest that the developed squid chitosan scaffolds might be further exploited for applications in cartilage tissue engineering.

1. Introduction

Marine organisms are the source of a large number of biopolymers with a wide range of properties that can be used for many different applications [1]. Despite this great potential, it has been observed a low exploitation of marine resources for high added-value ends [2], in particular considering the significant amounts of by-products being generated in fish processing plants and which major destiny is still animal feed. Indeed, in the particular case of squids, about 60% of the whole weight is being discarded (heads,

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viscera, pens, beaks, skin, etc.) [3]. Thus, the reuse and/or valorization of these materials, largely still regarded as residues, into novel materials with potential of application in different industrial sectors could have both economical and environmental interest. Considering the case of the giant squid *Dosidicus* gigas, calling attention by their unusually large size, pens (resulting from the processing of the organism to commercialize the mantle for food) can be a valuable source for the production of chitosan, with biomedical interest [2,4]. Chitosan is a natural polysaccharide obtained from the deacetylation of chitin, the second most abundant biopolymer, found in the exoskeleton of crustacean and endoskeleton of mollusks, besides the cell wall of some mushrooms [2,5–7], and the attention received from the biomedical community is mainly due to properties as cationic nature, mucoadhesion, hemostatic action, bactericide/fungicide activity, among others [5,7–12]. Most of the chitosan materials available commercially and consecutively the reported studies on their biomedical use are based of chitin extracted from readily available crustacean shells, which has a α crystallographic form [6,13–18]. This α-chitin is characterized by polymeric chains organized in anti-parallel way, with intermolecular hydrogen bonds, while the β-chitin obtained from squid pens exhibit parallel organization of the polymeric chains, with fewer or no intermolecular hydrogen bonds [4], resulting in polymer form more receptive to chemical modification [13,19–23], namely being easier to obtain a higher deacetylation degree [24], a parameter of chitosan ruling most of its biological properties. In fact, molecular weight and the degree of deacetylation of chitosan are influencing the biomedical performance of biomaterials based in this biopolymer, as is the case of transfection efficiency [25,26] or cell adhesion and proliferation [27–29]. Considering that squid chitosan has been already used to prepare porous scaffolds [2,17,30], including composites with calcium phosphates[17], nanofibers [31] and hydrogels [32], and motivated in one hand by the mentioned unusual large size of *D. gigas* pens and on the other hand by the promised economical (adding value to a by-product), environmental (tackling inappropriate waste disposal) and societal (proposing biomedical application) benefits, the present work proposes new freeze-dried chitosan scaffolds for soft tissue engineering, based in chitin isolated from *D. gigas* pens. We hypothesized that by varying freezing temperatures and degree of deacetylation of chitosan, scaffolds with different properties would be generated, from which a biomaterial with an adequate porosity envisaging the engineering of cartilage tissue would be produced. A commercially available chitosan from crab shells was used as a reference, characterized by a lower molecular weight. Mechanical properties, morphological features, and swelling/degradation profiles of chitosan scaffolds, as well as their capability to support ATDC5 chondrocyte-like cells culture were investigated.

2. Materials and methods

2.1. Material

Pens from giant squid *D. gigas*, from individuals captured in the cost of Chile and kindly provided by PESCANOVA, were used as raw material for β-chitin isolation. Chitosan from crab shells (C, Sigma-Aldrich, CAS 9012-76-4) was used after purification by a reprecipitation method [33]. All other reagents, of analytical grade, were used as received.

2.2. Isolation of β-chitin and further conversion in squid chitosan

β-chitin was isolated from squid pens of the species *D. gigas* using the methodology previously described [2]. Briefly, β-chitin was isolated through a deproteinization step of the grounded squid pens with a 1M sodium hydroxide (NaHO) aqueous solution, at a ratio 1:15 (w/v) 24 h, under stirring, at room temperature (RT). After, a deacetylation process was followed to convert β-chitin into squid chitosan (CS), through an alkaline treatment with a 50% NaHO solution at ratio 1:10 (w/v) under reflux, during 2 h. This process was repeated 3 times achieving a total reaction time of 8 h. Both CS and C were purified by a reprecipitation method [33]. The average molecular weight of C and CS was determined by using size exclusion chromatography – multiangle laser light scattering (SEC-MALLS) (Brookhaven Instruments Corporation, USA). The values obtained were 91.8 kDa for CS and 116 kDa for C. DD values of 76.6 ± 0.4% for C, and 91.2 ± 0.1% for CS were obtained using nuclear magnetic resource (NMR) spectroscopy (Bruker Avance II, 400 Hz, USA).

2.3. Preparation of chitosan scaffolds

Both CS and C powders were dissolved in 2% (v/v) acetic acid solution at a concentration of 4% (w/v) under stirring overnight. Chitosan solutions were then transferred into silicone molds and frozen at −80 °C (T1) or −196 °C (T2). The molds were then freeze-dried in freeze-dyrer (CryoDos -80, Telstar, Spain) during 4 days. Further, the structures were neutralized by immersion in ethanol/water solutions namely 50%, 70%, 80%, 90% and pure ethanol for 10 min each and followed by incubation in 1M NaHO during 30 min [34,35]. Subsequently, the structures were washed exhaustively with distilled water, frozen and freeze-dried. The chitosan structures were identified as CST1/CT1 when frozen at −80 °C and CST2/CT2 when frozen at −196 °C.

2.4. Characterization of the scaffolds

2.4.1. Mechanical testing

The mechanical behavior of developed scaffolds in the dry state was assessed under the static compressive solicitation. The unconfined static compressive mechanical properties of the scaffolds were measured using an INSTRON 5543 (Instron Int. Ltd., U.S.A.), with a compression load of 60% and cell of 1 kN, at a loading rate of 2 mm/min. The initial linear region on the stress/strain curves (n = 6), obtained by the secant method [36], defines the compressive modulus (or Young modulus).

2.4.2. Swelling by water uptake

Swelling behavior of the scaffolds was determined after immersion in solutions with three different pH values (pH5, pH7, and pH10), during 24 h, at 37 °C. The swollen samples weight was measured after removing the excess of surface water by gently tapping the surface with filter paper. The degree of swelling for each sample was calculated using Eq. (1).

$$Q = [(W_s - W_d)/W_d] \quad (1)$$

where W_s is the swollen sample weight under specified environmental conditions and W_d is dry sample weight.

2.4.3. Scanning electron microscopy (SEM)

The morphology of the scaffolds was analyzed using an XL 30 ESEM-FEG (Philips, US) scanning electron microscope. Longitudinal (cross-section) and superficial sections of the samples were obtained after freezing in liquid nitrogen. Prior SEM analysis, specimens were gold-coated using a sputter coater (Cressington, US) and the analysis was performed at an accelerating voltage of 15 kV and magnifications from 50× to 1000×.

2.4.4. Micro-computed Tomography (μ-CT)

The microstructure of the scaffolds was analyzed using a high-resolution μ-CT Skyscan 1072 scanner (Skyscan, Belgium) with a resolution of the pixel size of 8.7 μm and integration time of 1.9 s. The X-ray source was set at 32 keV and 191 μA. Approximately 500 projections were acquired over a rotation range of 180° with a rotation step of 0.45°. Data sets were reconstructed using standardized cone-beam reconstruction software (NRecon v1.4.3, SkyScan). The output format for each sample was 500 serial 1024 × 1024 bitmap images. A representative data set of 400 slices was segmented into binary images with a dynamic threshold of 55–255 (gray values). The same representative volume of interest (VOI) was analyzed for all the samples. These data sets were used for morphometric analysis (CT Analyser, v1.5.1.5, SkyScan) and to build the 3D models (ANT 3D creator, v2.4, SkyScan).

2.5. Biological assessment of developed chitosan scaffolds

2.5.1. Cell culture

Scaffolds with 4.36 mm in diameter, 5.73 mm in length, average values were sterilized by ethylene oxide prior biological tests. The scaffolds were placed in 48-wells tissue culture plates. Cells from the chondrogenic cell line ATDC-5 (ECACC, UK) at passage 3 were seeded at a concentration of 1×10^6 cells/scaffold. The cultures were maintained during 1, 7, 14 and 28 days in DMEM: F12 medium (Invitrogen, USA) supplemented with 10% FBS (Invitrogen, USA) and 1% antibiotic/antimycotic (Invitrogen, USA) at a temperature of 37 °C and 5% of CO_2. The medium was replaced every 2 days. For each one of the 3 independent experiments, three samples per material and per time point were used.

2.5.2. Metabolic activity analysis

The metabolic activity of cells cultured in the chitosan scaffolds was determined using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. Samples were incubated with an MTS solution, prepared using a 1:5 ratio of MTS reagent and DMEM without phenol red, 10% FBS and 1% ATB solution, for 3 h at 37 °C. The optical density (OD) was read at 490 nm, on a multiwell microplate reader (Bio-Tek Instruments, US). Results were normalized using dsDNA quantification values.

2.5.3. dsDNA quantification

dsDNA quantification was performed after each experimental time point using the PicoGreen Quantification Kit (Invitrogen Corporation, USA), according to the manufacturer instructions in a lysed cell suspension obtained after osmotic and thermal shocks. Fluorescence was read in a microplate reader at an excitation of 485/20 nm and emission of 528/20 nm.

2.6. Statistical analysis

Statistical analysis of the data was made by One-way ANOVA followed by Tukey posthoc test (compressive modulus and water uptake) or Two-way ANOVA followed by Bonferroni posthoc test (metabolic activity and dsDNA) to compare means, using Graph Pad Prism ® 5.0. Differences between the groups with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Scaffolds morphology and mechanical properties

The SEM micrographs showed that chitosan scaffolds have a lamellar structure, which was affected by the freezing temperature and by the chitosan source (Fig. 1a). Both CST2 and CT2 had smaller pore sizes than CST1 and CT1. CS scaffolds have higher average pore size than the ones obtained with C, at the same freezing temperature (Fig. 1a–c and Table 1). The overall analysis of the porosity obtained by micro-CT (Table 1) revealed that scaffolds processed at T1 have lower porosity than the ones processed at T2.

The mechanical properties of chitosan scaffolds were avaliated under compression tests, in dry conditions. The results of the compressive modulus (Fig. 2a) showed significantly higher values for CST2 scaffolds.

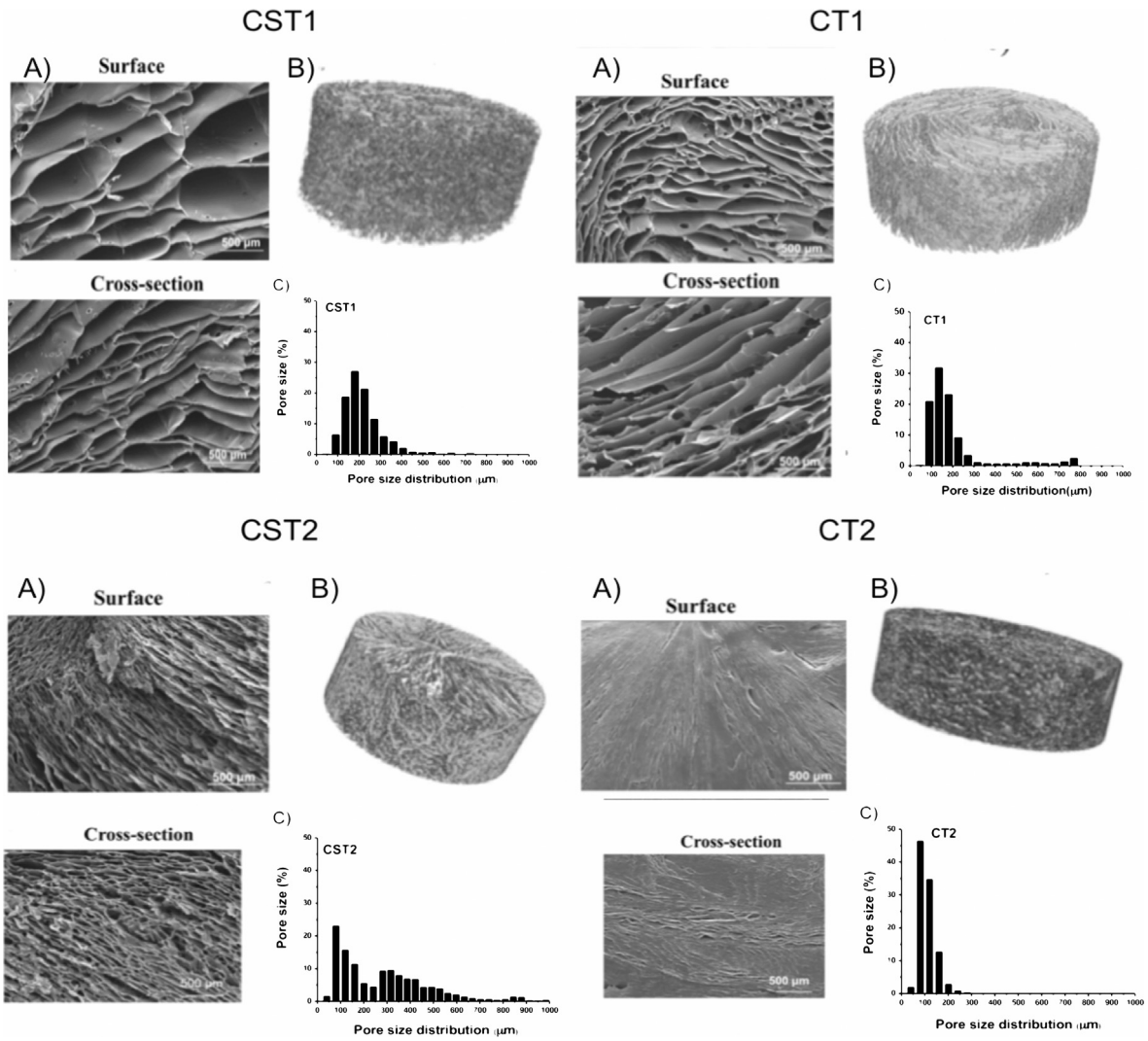

Fig. 1. (A) SEM micrographs of CST1, CST2, CT1 and CT2 scaffolds, (B) 3D images and (C) pore size distribution of scaffolds obtained by micro-CT analysis.

Table 1
Mean pore size and porosity of chitosan scaffolds determined by micro-CT analysis.

Samples	CST1	CST2	CT1	CT2
Mean pore size (μm)	251 ± 17	216 ± 17	196 ± 29	110 ± 24
Porosity (%)	69.7 ± 0.9	76.8 ± 3.9	62.4 ± 2.3	71 ± 12

3.2. Swelling tests

The swelling behavior of developed scaffolds was studied by evaluating the water uptake upon soaking in buffer solutions with different pH values (pH5, pH7, and pH10). In general, no significant differences were found between the scaffolds incubated in the same buffer solution or between equivalent scaffolds incubated in buffer solutions at different pH (Fig. 3), with uptake varying from 550% for CST1 at pH7 to 950% for CT2 at pH5 and pH10. Nevertheless, it seemed that the freezing temperature has a slight influence, with CST1 and CT1 samples exhibiting lower water uptake than the CST2 and CT2 counterparts, at the same pH.

3.3. Biological assessment

The SEM analysis of the constructs after 28 days of culture showed a high cellular density in all scaffolds (Fig. 4), confirming that ATDC5 chondrocyte-like cells were able to colonize the whole chitosan scaffolds. No significant differences were observed in the

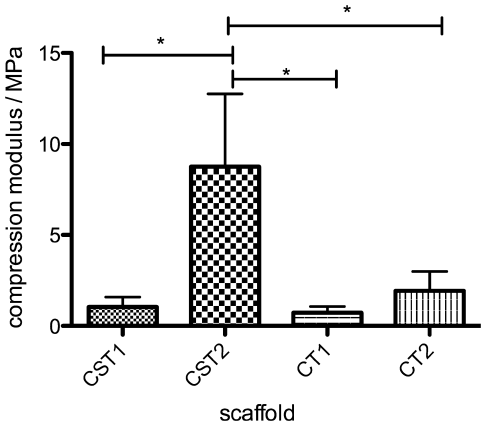

Fig. 2. (a) Compressive modulus of chitosan scaffolds (CST1, CST2, CT1 and CT2) determined from compression tests. The symbol * represents statistically significant differences, $p < 0.05$.

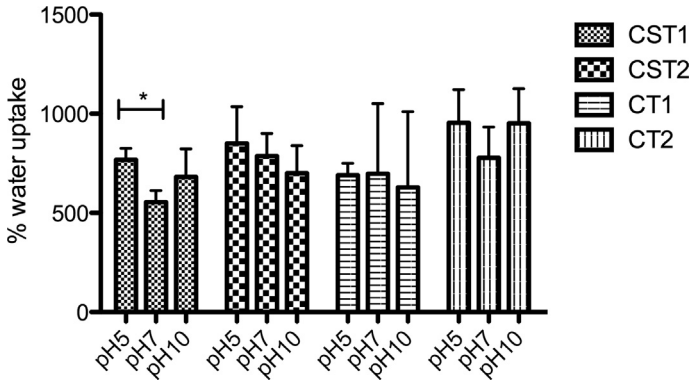

Fig. 3. Comparative swelling ratio of CS and C scaffolds after 24 h of immersion time in solutions with different pH values (pH5, pH7, and pH10). Data represent the mean ± standard deviation. The symbol * represents statistically significant differences, $p < 0.05$.

morphology of the cells, suggesting that neither the source of chitosan nor the freezing temperature affects cell morphology.

Fig. 5 shows the quantification of DNA (A), directly related to a number of cells, and respective cell metabolic activity (B), as a function of culture time. The results obtained showed that the DNA amount on all scaffolds significantly decreased from the first time point (1 day) to the following one, being approximately constant from day 7 on. The number of cells in CS scaffolds is systematically higher than on the C scaffolds processed at the same temperature. However, no tendency was observed for CS or the C scaffolds with the freezing temperature.

Regarding the cells metabolic activity normalized by the quantity of dsDNA, independently on the type of chitosan used or the processing temperature, it is constant up to 14 days and increased from day 14 to day 28.

4. Discussion

It is well known that freeze-drying is one of the most popular techniques for producing natural or synthetic polymers 3D porous scaffolds for tissue engineering. Scaffolds used for tissue engineering must have a porous architecture that allows tissue ingrowth and transport of nutrients and waste products [37,38]. By varying some of the parameters in freeze-drying tunable porous structures can be obtained. Factors such as shape and size of ice crystals, type of solvent and polymers, the concentration of the polymeric solution, freezing temperatures and speed of crystallization are known to affect the morphology and porous architecture of scaffolds [37]. For instance, Ouyang et al. [18] used liquid nitrogen to freeze cast composites of chitosan and carbon nanotubes obtaining radially aligned pores with 10 to 20 μm. In our results, the SEM analysis revealed a lamellar structure with larger pores for samples frozen at T1 and more compacted pores for samples frozen at T2. According to the literature [17,34,35], the morphology of CS and C scaffolds are related with freezing temperature, where the variation of ice crystals size can explain different morphologies and pore sizes [37]. In fact, samples frozen at T2 (−196 °C) were subjected to a faster cooling rate and ice crystals had no time to grow, resulting in smaller crystals but in a higher number. Thus, sublimation during freeze-drying originated smaller pore sizes, promoting the formation of a more compact porous structure. Xu et al. [37] reported that the nucleation temperature during a freezing process not only had a significant effect on the pore size and pore size distribution but also affect the overall level of porosity. Micro-CT analysis confirmed this tendency regarding mean pore sizes as well as porosity, which was highlighted for scaffolds processed at T2. Relating

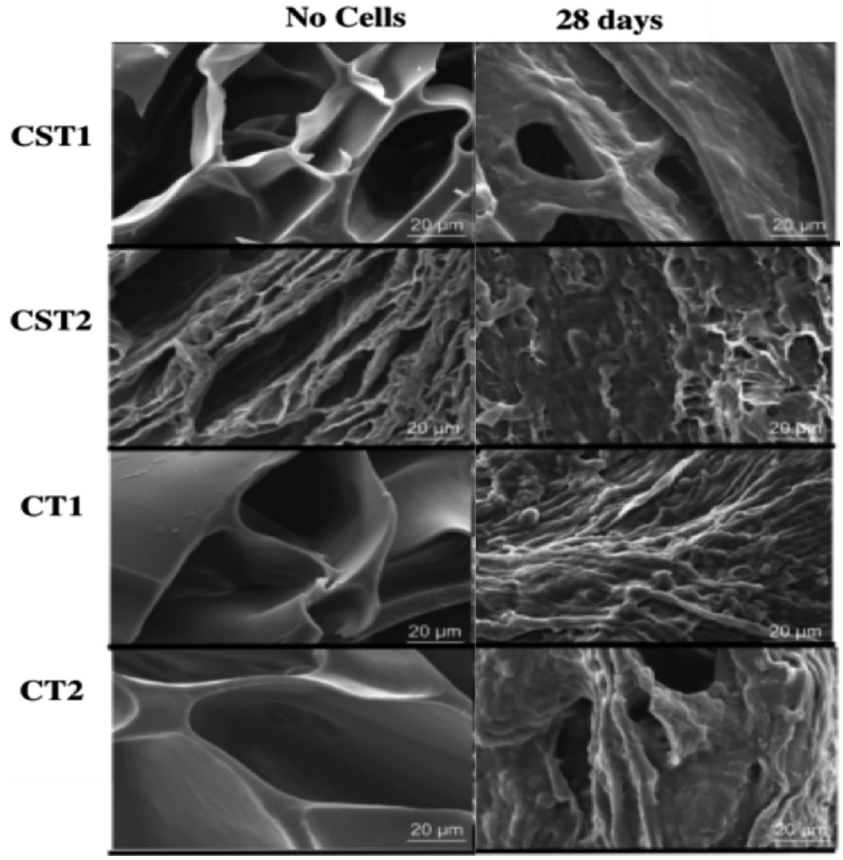

Fig. 4. SEM micrographs of ATDC5 cells cultured on chitosan scaffolds (CST1, CST2, CT1, and CT2) during 28 days. Scaffolds surfaces without cells were also analyzed as a reference.

to the type of chitosan used (characterized by different DD and M_w), CS scaffolds exhibited higher mean pore size and porosity, which can be related to the polymer M_w and viscosity of the solution, by the findings of other researchers with poly(vinyl alcohol) [39]. The formation of ice crystals during the freezing of an aqueous solution is dependent on the phenomenon of adsorption and desorption of the solute on the surface of the crystals being formed, in a dynamic process. Larger molecules such as polymers with higher molecular weight, as is the case of C in respect to CS, cover the ice crystals in a more irreversible way, which do not allow crystals grow further, thus leading to smaller pores upon sublimation. The neutralization method with ethanol/water can also influence the morphological features of the scaffolds, inducing higher porosity and smaller interconnectivity, probably due to the contact of the structures with ethanol [37].

The morphological features, namely the pore size and porosity discussed above, highly influence the mechanical properties of scaffolds [40]. Xu et al. [37] reported that the differences in pore arrangement, porosity, and pore size are the main factors that contribute to the variation of mechanical properties of the scaffolds. The compression modulus of scaffolds frozen at T2 seem to be higher than of those prepared at T1, which can be related the more compacted morphology and lower pore size exhibited by the T2 scaffolds. Shavandi et al. [17] have also evaluated the mechanical properties of chitosan-based scaffolds freeze casted using different temperatures, observing apparently opposite tendency. Nevertheless, in that study, the presence of calcium phosphates obtaining composite scaffolds would affect seriously the mechanical properties and the temperature range was not the same, from which a direct comparison is not appropriate.

Also, the DD of chitosan can affect the mechanical properties of the scaffolds. Nishino et al. reported that by varying the degree of deacetylation, the stiffness of chitosan may be affected [41], as well as crystallinity, from which a higher DD could lead to a structure exhibiting better mechanical properties [41]. In agreement with this, CST2 scaffolds, with higher DD (91.2%) appear to have higher compressive modulus than CT2, which has a DD of 76.65%.

To understand the behavior of the scaffolds at different pH, which is relevant for their applicability in biomedical applications, the swelling behavior of the scaffolds was assessed. The behavior of scaffolds was tested at pH 5 and 7 to simulated inflammatory and physiological conditions, respectively, and pH 10 was used to study the stability of chitosan in alkaline conditions [42]. Scaffolds frozen at T2 seemed to present higher water uptake, which can be attributed to the swelling of the more compact porous structures, in a plasticization effect of water over chitosan [43]. Moreover, the deacetylation degree does not seem to play a relevant role in the ability of the structures to uptake water. This result is supported by the absence of a clear influence of pH over the swelling of the

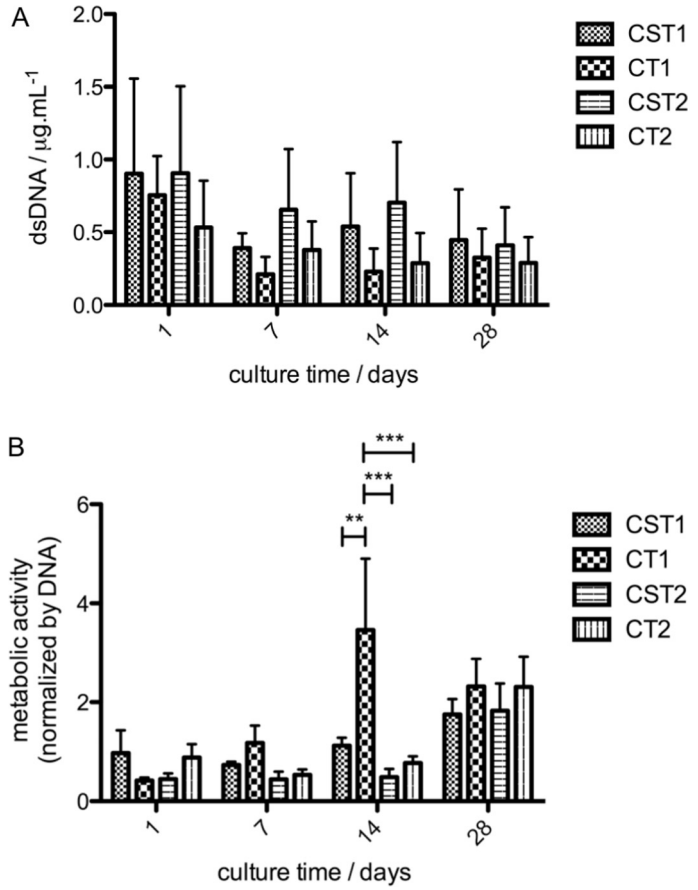

Fig. 5. (A) DNA content of ATDC5 cells on chitosan scaffolds, as a function of culture time; and (B) cellular metabolic activity, on CST1, CST2, CT1 and CT2 scaffolds, as the value of absorbance measured in MTS assay normalized by dsDNA content in the respective scaffold, as a function of culture time. The symbols ** and *** represent statistically significant differences, respectively for $p < 0.01$ and $p < 0.001$.

structures, which would be related with the protonation of free amines at low pH, more abundant in CS that has a higher deacetylation degree.

In the literature, some studies have attempted to use chitosan for cartilage repair, because it shares some characteristics with various GAGs naturally occurring in the human tissues [44]. The scaffolds should be designed to support cell adhesion and then tissues ingrowth, simultaneously allowing nutrient transfer from the outer surface to the core and cell metabolic residues from the interior to the exterior of the construct. In the current study, we investigate the adhesion and proliferation of ATDC5 chondrocyte-like cells on the developed chitosan-based scaffolds, as a preliminary approach to assess the potential of such structures for cartilage tissue engineering. The results obtained showed that the number of cells on all scaffolds decreased from day 1 to day 7, which is probably related to a significantly high number of cells initially cultured in the scaffold; most likely cells do not have sufficient surface to adhere and are therefore lost during subsequent medium changes. In order to better understand this behavior, cell distribution across the scaffolds was observed in histological images (supporting information) obtained upon hematoxylin staining, being observed that in the scaffolds processed with T2 the cells were predominantly adhered to the outer surface of the scaffolds, contrarily to CT1 and CST1 (processed at −80 °C), where the cells could be found on the interior of the scaffolds. Thus, it can be realized that although not affecting significantly the number of adhered cells, the more compact structure exhibited by chitosan scaffolds processed at T2 do not support cell migration to the interior of the scaffold, from which T1 is selected as the more appropriate freezing temperature to produce the scaffolds. This hypothesis is supported by the fact that from 7 up to 28 days of culture, the number of cells on the scaffolds inferred from the quantification of dsDNA seems to be stable. At this period of culture, CS scaffolds seem to support a higher cell number than their C counterparts, which can be related to differences on the DD. In fact, higher deacetylation degree represents a higher abundance of amine groups [45–47], which seems to promote a higher compatibility to chondrocytes. Chou et al. [48] also observe an influence of chitosan DD on cell proliferation, attributing it to an effect on surface roughness and stiffness, which are affected by changes in DD. Foster et al. [49] made similar observations with olfactory ensheathing cells cultured onto chitosan membranes with increasing DD. Interestingly, by assessing cells metabolic activity using the MTS assay, one could observe that the adhered cells became metabolically more active from day 7 to day 28 days. Thus, analyzing both dsDNA and MTS results, we can conclude that chitosan scaffolds can support the culture of chondrocytes. However, the high seeding density did not allow one to confirm significant variations among the conditions.

The present work adds to the state-of-the-art showing the relevance of squid chitosan as an alternative to the commercially available crab and shrimp chitosans for biomedical applications [32], calling the attention to the pivotal role of DD and polymer Mw on the performance of this biopolymer. In fact, when comparing results from different works, these features should always be considered, namely, when commercially available extracts are used, which face significant variation from batch to batch and sometimes the values provided by the manufacturer do not meet the ones determined by the researchers [32,49]. Adding the great difference in purity found between different chitosan providers and batches, particular care should be taken when a new sample needs to be selected for further use [50].

5. Conclusions

Scaffolds based on chitosan from crab shells and squid pens were produced by freeze-drying technique, using two freezing temperatures, namely −80 °C and −196 °C, obtaining very soft 3D porous structures. Squid chitosan scaffolds presented similar morphological features and swelling profile, but higher mechanical properties than the crab chitosan scaffolds, which could be attributed to the higher deacetylation degree of squid chitosan. Additionally, both chitosan scaffolds, when processed at −80 °C, support the ATDC5 chondrocyte-like cells adhesion and proliferation. Combining both results, squid chitosan scaffolds might be adequate to support cartilage regeneration, being thus interesting to proceed to a new study to evaluate the expression of chondrogenic markers and the formation of new cartilage-like tissue. The obtained findings revealed that endoskeletons of *Dosidicus gigas* could be used as a marine resource with environmental and economic advantages for the production of chitosan scaffolds in the alternative to the commercially common crabs and shrimps exoskeletons.

Acknowledgments

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