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Revealing the potential of squid chitosan-based structures for biomedical applications

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Abstract
In recent years, much attention has been given to different marine organisms, namely as potential sources of valuable materials with a vast range of properties and characteristics. In this work, β-chitin was isolated from the endoskeleton of the giant squid *Dosidicus gigas* and further deacetylated to produce chitosan. Then, the squid chitosan was processed into membranes and scaffolds using solvent casting and freeze-drying, respectively, to assess their potential biomedical application. The developed membranes have shown to be stiffer and less hydrophobic than those obtained with commercial chitosan. On the other hand, the morphological characterization of the developed scaffolds, by SEM and micro-computed tomography, revealed that the matrices were formed with a lamellar structure. The findings also indicated that the treatment with ethanol prior to neutralization with sodium hydroxide caused the formation of larger pores and loss of some lamellar features. The *in vitro* cell culture study has shown that all chitosan scaffolds exhibited a non-cytotoxic effect over the mouse fibroblast-like cell line, L929 cells. Thus, chitosan produced from the endoskeletons of the giant squid *Dosidicus gigas* has proven to be a valuable alternative to existing commercial materials when considering its use as biomaterial.

1. Introduction

The efficient exploitation of natural resources is a critical issue: on the one hand, the chemical and biological diversity of nature is uncountable, but on the other, natural resources are limited and must be fully exploited. From this perspective, the valorization of natural polymers is becoming increasingly important for technological and industrial-related applications [1], particularly polymers that can be obtained from marine organisms [2]. At the same time, great efforts into the research of natural polymers are being made in the biomedical field, due to their intrinsic properties and availability in nature [1].

Since 1990, biomedical studies on chitin (CT) and chitosan (CHT) have intensified due to their excellent biological properties such as biodegradation in the human body and immunological, antibacterial and wound-healing activity [3]. In particular, reports on applications such as drug delivery systems, wound dressings, tissue engineering, ophthalmology systems and others can be found in the literature [4–8].

CT, the second most abundant natural polymer after cellulose, is an ideally (1–4)-linked polysaccharide composed of 2-acetoamide-2-deoxy-β-D-glucopyranosyl residues, while CHT is its N-deacetylated derivative [9]. CT can be extracted from a wide range of natural sources such as crustaceans, fungi, insects, mollusks and some algae [10]. Depending on the source, it can exhibit one of two allotropic forms: α and β. For instance, α-chitin can be found in crustacean shells, while

β-chitin is present in squid pens [11]. So far, little attention has been given to the β-crystallographic form, mainly due to the difficulty in obtaining the product; because of this CHT has been commercially produced almost exclusively from α-chitin [10, 11]. Despite the above-mentioned properties that make them quite interesting biomedically, both CT and CHT exhibit limiting factors in their reactivity and processability; they are not water soluble, degrade thermally and it is difficult to convert CT into CHT by deacetylation [11, 12]. β-chitin has weaker intermolecular bonds, higher reactivity and higher affinity for solvents when compared to α-chitin. Besides this, CHT prepared from β-chitin also exhibits higher reactivity and significant bactericidal activity [10, 13]. Thus, CHT derived from β-chitin obtained from squid pens may overcome some of the aforementioned drawbacks. Moreover, the increase in the valorization of marine resources is resulting in the increase of the raw material for β-chitin production. In fact the waste from food processing industries, namely the endoskeletons of cephalopods, could be both economically and environmentally valuable asa resource for obtaining CT. The aim of the present work consists of the isolation and the physicochemical and biological characterization of β-chitin obtained from the squid pen of *Dosidicus gigas* (a giant squid). The CT was isolated from the mentioned squid specie using the method based on the work of Chaussard and Domard [14], who evaluated the various physicochemical parameters that can influence the production of CT from a sample of squid pens (*loligo)*. Further, the extracted β-CT was converted to CHT through a deacetylation reaction. Then, squid chitosan (CHTS) was processed as membranes and scaffolds using the solvent casting and freeze-drying techniques, respectively. These matrices were characterized by their mechanical properties, swelling behaviour, structural stability and cytotoxicity.

2. Materials and methods

2.1. Materials

Squid pens from *Dosidicus gigas*, used as raw material for β-chitin isolation, were obtained from individuals captured on the coast of Chile and kindly provided by PESCANOVA. Commercial chitosan (CHTC, Sigma Aldrich, CAS 9012-76-4) with medium molecular weight was used after purification. Commercial chitin (CTC, Sigma Aldrich) was used as received. All other reagents, of analytical grade, were used as received.

2.2. Production of squid chitosan

2.2.1. Isolation of chitin from squid pens. Isolation of β-chitin from squid pens of the species *Dosidicus gigas* was performed using a method based on the work of Chaussard and Domard [14]. In brief, the isolation of chitin (squid chitin (CTS)) consisted of washing, grinding and deproteinization. Firstly, squid pens were washed with tap water and ground into particles 1 mm in diameter. Then, the particles were submitted to deproteinization by treatment with 1 M sodium hydroxide (NaOH) (Panreac Química SAU, Spain) aqueous solution, at a ratio of 1:15 (w/v), for 24 h (hrs), under stirring, at room temperature (RT). The obtained material was filtered under vacuum and washed several times with distilled water until neutrality was reached, then dried at RT.

2.2.2. Conversion of chitin into chitosan. A deacetylation process was used to convert CTS into CHTS based on the work of Shimojoh *et al* [10], consisting of the hydrolysis of the acetamide groups of CT by alkaline treatment with a 50% NaOH solution, at a ratio of 1:10 (w/v), under reflux between 85 to 100 °C, over 2 h. This alkaline treatment was repeated three times, where the NaOH solution was replaced each time. Afterwards, the material was filtered and washed until neutral. Finally, the CHTS was frozen at −80 °C and freeze-dried (Telstar equipment, Spain) for four days [9].

2.2.3. Purification of chitosan. A purification process was necessary to obtain pure CHT which was used for the production of CHT-based materials such as membranes and scaffolds [15]. Both CHTS and CHTC were purified by a reprecipitation method [15]. CHTS and CHTC were dissolved at concentration of 1% (w/v) in 2% (v/v) acetic acid (from Pronolab, Portugal) aqueous solution. The system was maintained and stirred overnight at RT. Afterwards, the solution was filtered twice to remove impurities and CHT was then precipitated using 3 M NaOH solution. The precipitate was separated by filtration, washed with distilled water until neutral and freeze-dried for four days.

2.3. Characterization of chitin/chitosan

2.3.1. Determination of molecular weight. The molecular weight of CHTS and CHTC was determined by size exclusion chromatography-multiangle laser-light scattering (SEC-MALLS) [16]. The system was composed of an Iso Pump G1310A Hewlett Packard, with light scattering PSS SLD7000 MALLS detector (Brookhaven Instruments Corporation, USA) operating at 660 nm. Both CHTS and CHTC were dissolved in acetic acid solutions of 0.3 to 2 g L^{-1} and shaken at RT. CHT solutions were filtered with a syringe filter membrane of 0.45 μm. Analyses were performed at RT in 0.2 M acetic acid buffer solutions and 0.15 M ammonium acetate solution as a mobile phase. Values of dn/dC were taken from the literature [9, 16].

2.3.2. Determination of particle size. The particle size of CT and CHT was determined by dynamic light scattering (DLS) on a suspension of those materials in water, using Zetasizer® equipment (Malvern, UK).

2.3.3. Measurement of the degree of N-deacetylation. The average degree of deacetylation (DD) of the CT and CHT samples was determined from data of elemental analysis and nuclear magnetic resonance spectroscopy (NMR), respectively. Elemental analysis was performed in the Laboratory Analisis Instrumental, (CACTI), University of Vigo, using a Fisons EA-1108 elemental analyser. NMR spectra were recorded using Bruker Avance II 400 Hz. For this purpose, the samples were dissolved in 2% deuterated acetic

acid in deuterated water (D_2O) solution. After dissolution approximately 1 mL of the CHT sample solution was transferred to a 5 mm NMR tube. The sample tube was inserted in a magnet and allowed to reach thermal equilibrium 10 min before the experiment was performed.

2.3.4. Chemical composition. The chemical composition was characterized by Fourier transform infrared spectroscopy (FTIR), using a Shimadzu IR Prestige 21 spectrometer (from Shimadzu, Europa), in the spectra region of 4000–400 cm^{-1} with resolution of 16 cm^{-1} after acquisition of 32 scans. The samples were powdered, mixed with potassium bromide (KBr) and processed into pellets.

2.4. Preparation of polymeric systems with squid and commercial chitosan

Polymeric systems, in particular membranes and porous structures, were produced using squid and CHTC. The CHTC was used as reference.

2.4.1. Production of membranes. CHT membranes were prepared by solvent casting [17]. CHTS and CHTC (powder) were dissolved in 2% (v/v) acetic acid solution at a concentration of 1% (w/v). Then, CHT solutions were poured into Petri dishes and the solvent allowed to evaporate at RT for about four days. In order to neutralize the acetic acid, the dried membranes were immersed in 0.1 M NaOH solution for about 15 min [18] and then washed with distilled water to remove all traces of alkali, followed by drying at RT.

2.4.2. Production of porous structures. Porous CHT structures were developed using the freeze-drying technique [5, 19]. CHTC and CHTS powders were dissolved in 2% (v/v) acetic acid solution at concentrations of 3 and 4% (w/v). CHT solutions were then transferred into a silicone mould, frozen at –80°C overnight and freeze-dried for four days to completely remove the solvent, thus producing the desired porous structures. The neutralization of scaffolds was performed using two different methods. In the first method (M1) the samples were immersed in 1 M NaOH for one hour and washed with distilled water until neutral [20]. In the second method (M2) [20], the samples were soaked in ethanol/water (Et/W) solutions for 10 min. After that, the scaffolds were immersed in 1 M NaOH solution for 30 min, and then washed abundantly with distilled water until neutral [21]. The samples were identified as CHTXY, where X indicates the polymer concentration and Y the method of neutralization.

2.5. Characterization of polymeric systems

2.5.1. Mechanical properties. The mechanical properties of the CHTS and CHTC membranes were characterized by dynamic mechanical analysis, DMA. The viscoelastic measurements were performed using a TRITEC8000B DMA from Triton Technology [10], equipped with the tensile mode. The experiments were performed in wet and dry states, at 37 °C. The thickness of the tested membranes (in the dry state) was: 30.0 ± 2.1 μm and 32.0 ± 2.9 μm for CHTS and CHTC, respectively. The tests were also performed in wet conditions using phosphate buffer saline solution (PBS) and the registered values for thickness were: 60.3 ± 1.9 μm and 42.7 ± 2.1 μm for CHTS and CHTC, respectively. The distance between the clamps was 10 mm and the membranes samples were cut to about 5 mm widths, measured accurately for each sample. Four samples were used in each analysis. All samples were analysed in wet conditions, using PBS, placed in a Teflon reservoir, with prior membranes equilibrated for about 50 min in PBS solution. After equilibration at 37 °C, the DMA spectra were obtained during a frequency scan between 0.1 and 20 Hz. The experiments were performed under constant strain amplitude (30 μm). A static pre-load of 1 N was applied during the tests to keep the sample tight. The same conditions were used for the dry experiments, but the membranes were not immersed in the liquid bath. The DMA results were presented in terms of two main parameters: storage modulus (E') and loss factor (tan δ).

The mechanical behaviour of the developed scaffolds in the dry state was assessed under static compressive solicitation. The unconfined static compressive mechanical properties of the scaffolds were measured using an INSTRON 5543 (Instron Int. Ltd, USA) with a load cell of 1 kN for 60% of strain, at a loading rate of 2 mm min^{-1}. The initial linear modulus on the stress/strain curves ($n = 6$), obtained by the secant method, defines the compressive modulus (or Young's modulus).

2.5.2. Structural stability. Structural stability tests were performed by immersing both squid and CHTC membranes and scaffolds in PBS solution (pH 7.4) and PBS with 13.6 mg L^{-1} lysozyme (Sigma Aldrich, Germany), at 37 °C, for up to 60 days, changing the solution every seven days. At the end of each period of time (time point), the samples were removed and washed thoroughly with distilled water. Finally the membranes were dried at 60 °C and the weight loss was calculated using equation (1):

$$\text{Weigh loss} = \left[\left(\frac{W_i - W_f}{W_f}\right) \times 100\right] \quad (1)$$

where W_i is the initial weight and W_f is the final weight of the sample.

2.5.3. Surface properties. The surface properties of the CHTS and CHTC membranes were assessed by means of static contact angle (θ) measurements using the sessile drop method with ultra-pure distilled water (polar) and diiodomethane (non-polar) (OCA equipment, Germany and SCA-20 software), at RT (ca 20 °C). At least five measurements were performed for each solvent. Surface tension (σ), as well as its polar (σ_p) and dispersive (σ_d) components, were determined by the Owens and Wendt method [22], using the software G402.

2.5.4. Study of morphology: micro-computed tomography (μ-ct). The microstructure of the scaffolds was evaluated using a high-resolution μ-ct Skyscan 1072 scanner (Skyscan, Kontich, Belgium) with a resolution pixel size of 6.59 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 500 slices was segmented into binary images with a dynamic threshold of 57–255 (grey values). The same representative volume of interest (VOI) was analysed 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).

2.5.5. Study of morphology: scanning electron microscopy. The morphology of the scaffolds, before and after the neutralization process, was also observed using a XL 30 ESEM-FEG Philips microscope. Longitudinal sections of samples were obtained after being frozen in liquid nitrogen. Prior to SEM analysis, specimens were coated with gold using a Cressington sputter coater and the analysis was performed at an accelerating voltage of 15 kV and magnifications from 50 × to 100 × .

2.5.6. Cytotoxicity tests. In order to assess the possible cytotoxicity of the samples, extracts (leachables) of all membranes and scaffolds were evaluated following MEM extraction tests (72 h), according to ANSI/AAMI/ISO-10993-5 guidelines [23]. For these tests a mouse fibroblast-like cell line, L929 cells, (L929 cells; ECACC, UK) was used and cell metabolic activity was assessed by using a MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. In MEM extraction tests, the materials and the control (latex as positive control) were extracted by incubation in complete medium (the same used for cell culture) for 24 h at 37 °C and 60 rpm (in thermostatic bath) [24]. After the defined period of incubation, the resulting solution was removed, thus obtaining the extraction fluid. L929 cells were cultured in basic medium: Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, USA) with phenol red and supplemented with 10% foetal bovine serum, FBS (Gibco, UK) and 1% antibiotic/antimycotic (A/B, Gibco, UK) solution and incubated at 37°C in an atmosphere containing 5% CO_2 until achieving 90% confluence. Then, a cell suspension was prepared with a concentration of 1×10^5 cells mL^{-1} and seeded on extracts in 24-well plates and incubated for 24, 48 and 72 h. The L929 cells' relative viability (%) was determined for each extract and compared to tissue culture polystyrene (TCPS), used as a negative control of cell death. Latex extracts were considered as a positive control of cellular death. After each time point extracts of the solutions were removed and the plates were evaluated microscopically (in an inverted phase contrast optical microscope) to assess the confluence of the monolayer and changes in cellular morphology. At each point an MTS test was performed to assess L929 cellular viability. For this assay, an MTS solution was prepared using a 1:5 ratio of MTS reagent and culture medium containing DMEM without phenol red, 10% FBS and 1% A/B solution, followed by a 3 h incubation period at 37 °C. Finally, the optical density (OD) at 490 nm, directly proportional to the concentration of a brown formazan product resulting from the bioreduction of MTS by living cells in culture, was read on a multiwell microplate reader (Synergy HT, Bio-Tek Instruments). All cytotoxicity-screening tests were performed using nine replicates ($n = 9$). The obtained results were compared with controls, namely culture polystyrene (TCPS) as a negative control and latex as a positive control of cellular death.

2.6. Statistical analysis

Statistic analysis of the data was performed by hypothesis testing *t*-test using OriginPro 8.0, OriginLab Corporation. Differences between the groups with $p < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Chitosan produced from squid pens

Despite the increasing attention that marine organisms have been receiving, they are efficiently exploited and residues with valuable compounds are being discarded. An example is the endoskeleton of squid from which CT and consecutively CHT can be obtained. CT was isolated from the endoskeletons of *Dosidicus gigas* (giant squid) by deproteinization with NaOH solution, with a yield of 38.6% in respect to raw material. Further, CTS was converted into CHTS by a deacetylation procedure, as described in the experimental section and several materials were characterized by FTIR. The spectra, in figure 1(*A*), obtained with CTC and CTS, showed the characteristic peaks of chitin at 1660 cm^{-1} (amide I) and 1580 cm^{-1} (amide II) [11], which indicated that independently of the source, chitin (CTS and CTC) have the same chemical nature. When comparing the spectra obtained with CHTC and CHTS, in figure 1(*B*), their similarity is noted, where the characteristic peaks of CHT were observed at 1660 cm^{-1} (amide I) and 1580 cm^{-1} (amide II) [12], but with different relative intensities than the ones observed in chitin spectra, as highlighted in figure 1(*C*). In particular, the peak observed at 1660 cm^{-1}, attributed to the vibration of C–O bonds, is more intense for CT than for CHT because during the deacetylation of chitin C–O groups are being removed from the polymer. These findings support the success of the used procedures on the production of chitin and CHT from endoskeletons of *Dosidicus gigas*.

The performance of CT and CHT in several applications, namely in biomedicine and pharmacy, is influenced by properties such as the size of the polymer chains and the DD [25]. Thus, in order to assess those properties, the average molecular weight of CHTC and CHTS was determined by using SEC-MALLS, which obtained a value of 91.8 kDa for CHTS and 116 kDa for CHTC. Both CTC and CTS were not analysed by liquid chromatography due to their difficult solubilities.

The particle sizes of all the developed materials were determined by dynamic light scattering and values of 282 nm for CTC, 304 nm for CTS, 145 nm for CHTC and 149 nm

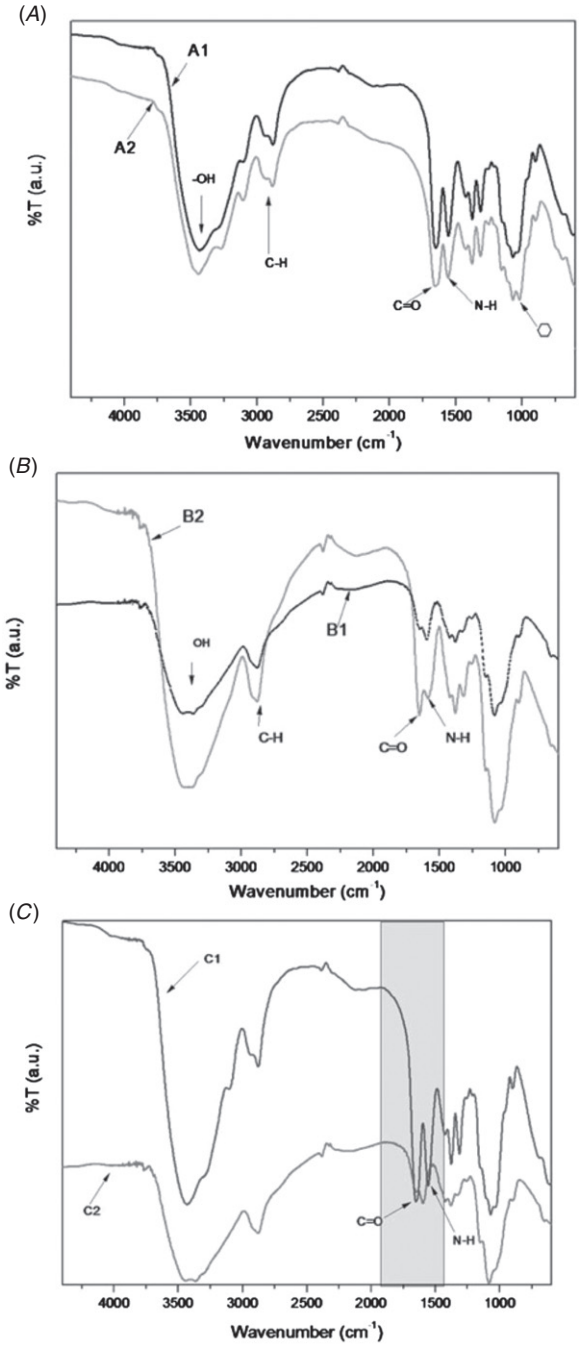

Figure 1. FTIR spectra of (*A*) chitin (CT), A1-CTS, A2-CTC (*B*) chitosan (CHT), B1-CHTS, B2-CHTC, (*C*) C1-CTS and C2-CHTS.

for CHTS were obtained. By these results, CT samples presented a particle size almost the double the one observed for CHT samples. The particle size measured by dynamic light scattering is related to the hydrodynamic volume of the polymer particles, which is dependent on the charge and conformation of the polymer in aqueous suspension [26]. In fact, it can be hypothesized that polymers with higher molecular weights may result in particles with higher hydrodynamic volumes. Thus, one can speculate that CHT has a significantly smaller molecular weight than CT, which is in agreement with the structural stability of the polymer that may be expected to occur together with the removal of acetyl groups during the chemically aggressive deacetylation process.

The DD, which is the number of glucosamine units of the biopolymer chain with respect to the total number of units [27], is an important property of CHT that dictates some of its physicochemical properties such as solubility, crystallinity, swelling behaviour and material degradation [25]. The obtained DD of the samples were: 42.1 ± 0.1 for CTC, 7.2 ± 0.1 for CTS, 76.6 ± 0.4 for CHTC and 91.2 ± 0.1 for CHTS. Both CTC and CTS samples have different values of DD, which can be attributed to the different sources and respective types of CT (crustaceans have α-chitin and mollusks have β-chitin), but also to the isolation processes used in each case. Furthermore, alkaline treatments used for deproteinization can also result in the removal of some acetyl groups and thus increase the DD value. In fact, CTC production follows an industrial-scale process involving a substantial amount of raw material, so control of the process can be difficult. However, the production of CTS following a laboratory-based methodology allows better control of all processing steps [28]. To support this statement, the CHTS has a DD higher than the ones obtained for CHTC. According to the literature [11], both α-chitin and β-chitin show differences in intermolecular forces, where β-chitin has weaker intermolecular forces. This fact reinforces the susceptibility of β-chitin to the deacetylation reaction and consequently explains its high DD.

3.2. Chitosan membranes

After the successful production of CHT from squid pens, its potential for biomedical applications was assessed through the production and characterization of membranes and porous structures.

CHT membranes were prepared by solvent casting, as described in detail in the Materials and Methods section, and their mechanical properties were studied by DMA. Figure 2 presents the viscoelastic behaviour of membranes prepared with CHTC and CHTS, analysed in dry (A) and wet (B) states. The storage (elastic) modulus (E') of all studied membranes increased with increasing frequency, with membranes made with CHTS exhibiting the highest E' values [4, 29]. This suggests that these membranes possess a higher stiffness than those prepared with CHTC. Figure 2 also presents the variation of the phase angle through tan δ, with the frequency, that measures the damping properties of the samples and provides an indication of its viscoelastic characteristics [30]. It is observed that both types of membranes present the same behaviour in tan δ. Since tan δ represents the ratio between E'' and E', it can be concluded that the loss modulus was also higher for the membranes prepared with CHTS [31]. Thus, these membranes have also a higher viscous behaviour. Comparison between the results obtained in wet conditions indicated that membranes prepared with CHTS have higher mechanical properties than ones observed on membranes prepared with CHTC. Overall, the values were lower than the ones observed in dry conditions, suggesting that membranes were less stiff in wet conditions probably due

Table 1. Contact angles (θ); dispersive (γ_d) and polar (γ_p) components and superficial energy (γ) of the developed membranes (CHTS and CHTS).

Material		θ_{water}(°)	$\theta_{diiodomethane}$(°)	γ_d (mN m^{-1})	γ_p (mN m^{-1})	γ (mN m^{-1})
CHT	CHTC	99.8 ± 7.6	61.1 ± 3.3	26.2 ± 1.4	2.0 ± 0.7	28.2 ± 1.6
	CHTS	85.5 ± 1.1	55.2 ± 1.7	26.8 ± 0.9	4.7 ± 0.7	31.4 ± 1.1

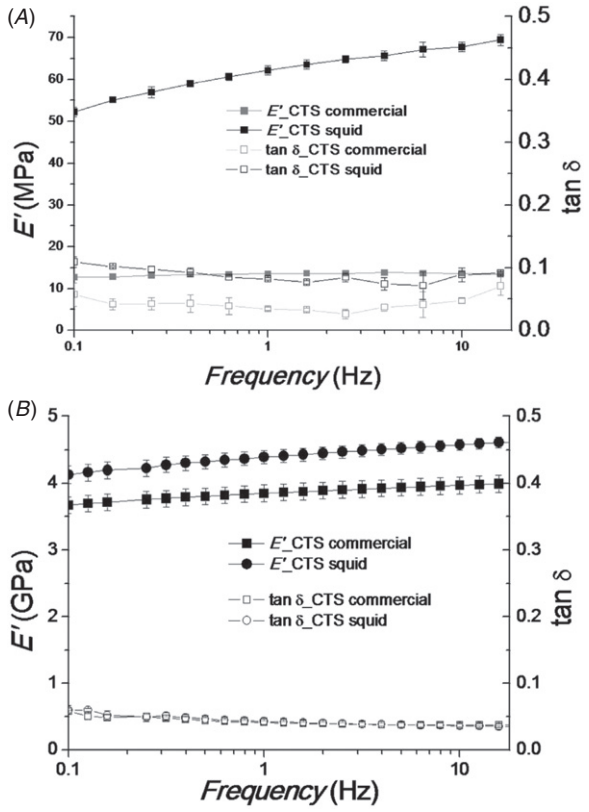

Figure 2. DMA results of CHTS and CHTC membranes: (*A*) wet state (*B*) dry state.

to the plasticization effect of water [29, 31]. Regarding viscous behaviour, since tan δ was higher for CHTS membranes, the differences in the loss modulus of both membranes should be less pronounced, but the membranes prepared with CHTS were still presenting higher viscous behaviour than the ones prepared with CHTC. These observations are in agreement with others of mechanical strength with DD (between 60 and 100), on CHT swollen with PBS [32].

The surface energy of the prepared CHT membranes was also assessed, in order to characterize their hydrophilicity. The surface energy and contact angles of membranes prepared with CHTS and CHTC were measured by using a contact angle goniometer and the obtained results are summarized in table 1. It can be seen that the CHTS membranes are less hydrophobic than the CHTC membranes. This behaviour is attributed to the higher deacetylation degree of CHTS, since the amino groups have more affinity for water than acetylamine ($NHCOCH_3$) and the number of amino groups was higher in CHTS than in CHTC. The surface energy of CHTS membranes was higher than CHTC membranes. However, no significant changes were seen for the non-polar and polar components of surface energy [1].

Table 2. Weight loss of CHTS and CHTC membranes in PBS, in the presence or absence of lysozyme, after 3 to 60 days of incubation at 37 °C.

Time (days)	CHTS[a]	CHTS[b]	CHTC[a]	CHTC[b]
3	7.6 ± 1.3	9.0 ± 3.8	18.3 ± 2.4	15.1 ± 1.5
7	5.2 ± 0.7	8.4 ± 1.4	17.2 ± 3.7	15.7 ± 3.5
14	4.8 ± 1.4	7.8 ± 2.0	17.0 ± 1.3	11.5 ± 2.9
21	5.3 ± 1.6	8.2 ± 1.5	11.7 ± 3.7	12.5 ± 4.8
28	6.6 ± 0.2	8.1 ± 2.5	10.9 ± 2.5	11.9 ± 6.4
45	6.6 ± 0.4	9.2 ± 0.9	10.6 ± 0.8	11.7 ± 1.2
60	2.9 ± 0.6	4.8 ± 0.4	11.3 ± 2.5	12.2 ± 4.7

[a] The samples were immersed in PBS.
[b] The samples were immersed in lysozyme (13.6 mg L^{-1}).

Membranes for tissue engineering, depending on their composition, are usually expected to degrade after successful tissue regeneration [33]. In this way, it is critical to study the structural stability profile of produced CHT membranes in order to better characterize their potential for application in tissue engineering strategies. The aim of the performed structural stability studies was to simulate physiological conditions using the enzymes present in human serum that would be responsible for enzymatic hydrolysis of CHT materials *in vivo*, in order to further control and tailor the structural stability of CHT membranes [34]. *In vivo*, CHT is degraded by enzymatic hydrolysis with lysozyme being the primary agent. This enzyme is present in human serum with a concentration in the range of 7–13 mg L^{-1} [35]. From this perspective, CHTS and CHTC membranes were incubated in PBS, in the absence (control) and presence of lysozyme, for up to 60 days. The results are shown in table 2. The structural stability profile seems to be stable for at least 60 days of incubation. According to these findings, CHTC membranes are more susceptible to enzymatic structural change, which is most likely due to smaller DD, since the structural stability of CHT appears to be inversely related to the DD [35]. From this perspective, membranes prepared with CHTS are suitable for drug delivery patches and as wound dressings, due to its smaller structural stability rate, which would make these CHT membranes more durable.

3.3. Porous chitosan structures

As already mentioned, the potential of CHTS for biomedical applications was also assessed through the production and characterization of porous CHT structures. These porous structures were prepared by freeze-drying CHT solutions with different concentrations, followed by further neutralization with one of two methods, namely NaOH solution (M1) and ethanol/water prior to treatment with NaOH solution (M2).

The morphology of porous structures is an important parameter to be considered when an application as scaffold

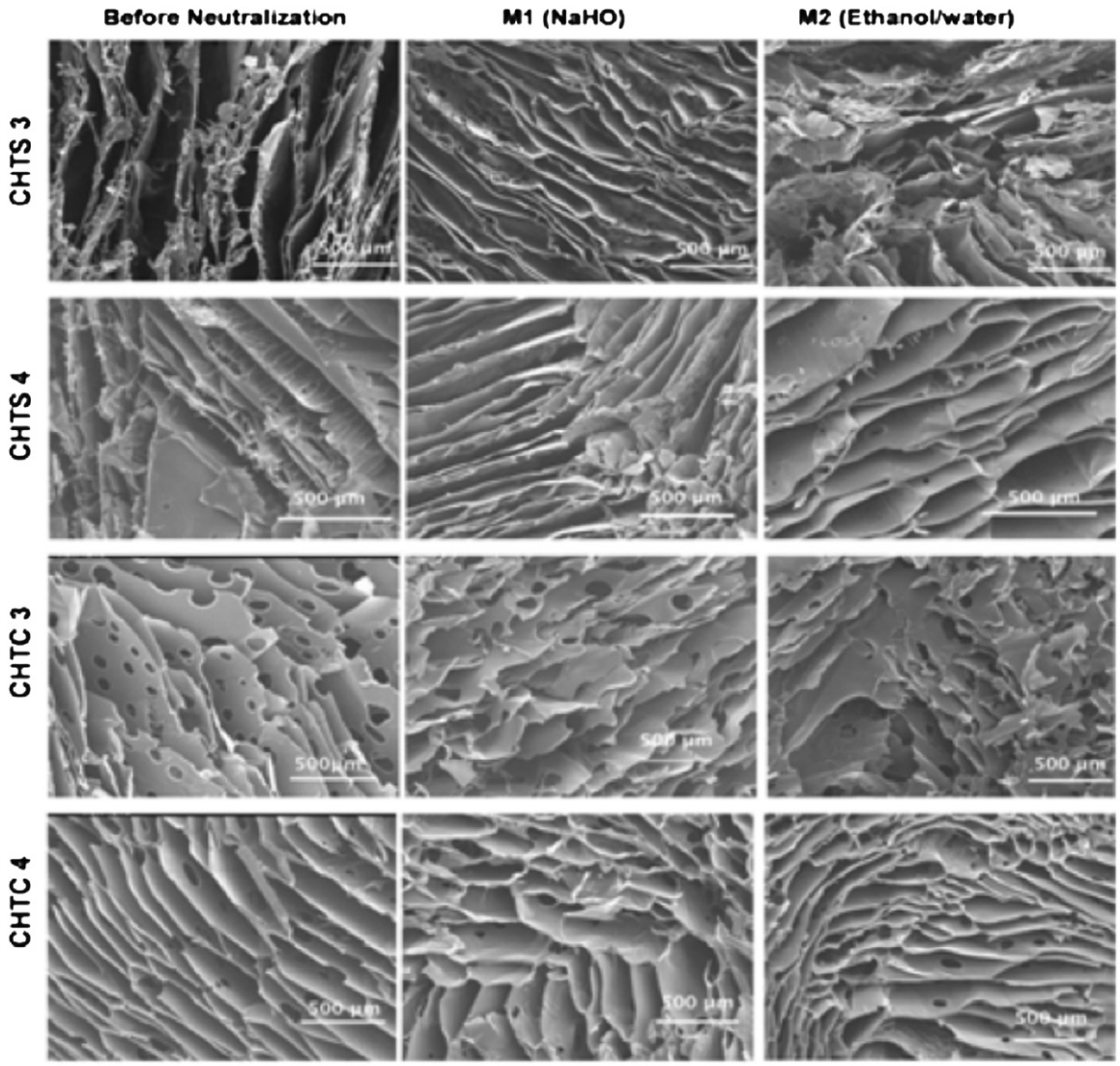

Figure 3. SEM microphotographs of scaffolds prepared with commercial and squid chitosan, using 3% or 4% solutions, illustrating their morphology before and after neutralization with method 1—M1 (NaOH) or method 2—M2 (ethanol/water and NaOH).

for tissue engineering is envisaged. Those structures should exhibit a porosity and pore interconnectivity that allows not only the successful seeding of cells, but also their proliferation into the structure [20]. In order to assess the morphology of the prepared CHT scaffolds, SEM analysis has been performed and the obtained images are illustrated in figure 3. Those images show that all scaffolds have well-defined morphology with lamellar features. According to the literature [20, 21], CHT based materials need to be neutralized in order to stabilize their structure, and several methods have been proposed, involving the use of ammonia solutions, NaOH solutions [20], ethanol [20] or mixtures of NaOH solutions with ethanol [20]. As can be observed in the images depicted in figure 3, before neutralization there are no significant changes in the morphology of CHT scaffolds. Moreover, after neutralization with M1, the lamellar structure was kept constant for all CHT scaffolds. However, when using M2 for the neutralization of scaffolds the lamellar structure was partially destroyed on the scaffolds prepared with CHTC 3 and CHTS 3 (scaffolds prepared with CHTC and CHTS respectively, with concentrations of 3%) and more significantly when using CHTS. The observed morphological changes are probably due to partial dissolution of the scaffold after contact with ethanol. Regarding the scaffolds prepared from CHT 4 solutions, no significant changes in the lamellar morphology are observed, although when using CHTS 4 the presence of larger pores in the range of 500 to 1000 μm was noted. As discussed before, these morphological changes observed in CHTS scaffolds may be due to partial dissolution and a consequential loss of structure, which is in agreement with the expected higher reactivity and interaction of CHTS with solvents reported in the literature [20]. However, the high polymer concentration used in the production of these scaffolds (CHTS 4 and CHTC 4) could also contribute to these results. Overall, freeze-drying CHT solutions conjugated with one of the described neutralization methods can be used to produce porous CHT structures with lamellar features and pore sizes ranging from 200 μm to 1000 μm, which are adequate for tissue engineering approaches [36].

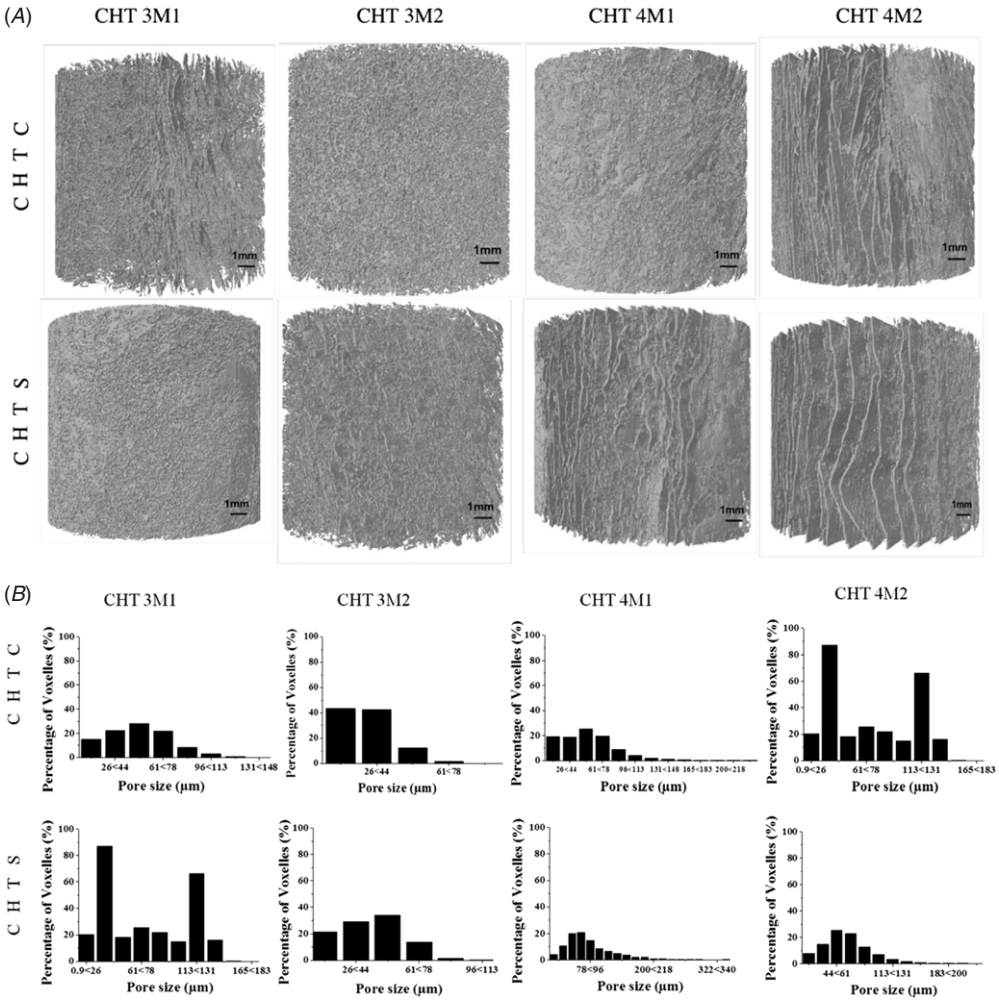

Figure 4. (*A*) μ-ct 3D microphotographs of scaffolds and (*B*) mean pore size and distribution of pore size, observed after neutralization.

Table 3. Values of interconnectivity, pore size and porosity for CHTS and CHTC scaffolds before and after neutralization.

Samples	Properties	CHT 3[a]	CHT3[b]	CHT3[c]	CHT4[a]	CHT4[b]	CHT4[c]
CHTC	Interconnectivity (%)	88.8	81.9	87.1	3.69	68.2	36.1
	Pore size (μm)	151.43	52.1	30.2	54.32	54.7	84.1
	Porosity (%)	67.9	57.3	62.0	78.30	66.4	73.1
CHTS	Interconnectivity (%)	2.10	73.3	93.7	1.83	50.3	60.3
	Pore size (μm)	48.54	65.1	42.7	53.55	85.2	67.1
	Porosity (%)	65.66	64.3	41.8	65.87	71.2	71.9

[a] Samples before neutralization.
[b] Samples neutralized by M1 (NaOH).
[c] Samples neutralized by M2 (ethanol/water).

Morphological analysis of the developed CHT scaffolds was also assessed by μ-ct, particularly to characterize pore size, porosity and pore interconnectivity. The 3D μ-ct images, obtained from digital geometry processing from a series of two-dimensional x-ray images, are illustrated in figure 4. Table 3 also presents the determined values of interconnectivity, porosity and pore size for the studied CHT scaffolds. Upon observation of the images of the CHT scaffolds obtained from μ-ct analysis, the porosity seems to be similar for different types and CHT concentrations. From the determined values, it can be stated that scaffolds prepared with both CHTs have higher porosity with increasing concentration of CHT solution, which can be explained by differences in the formation of ice crystals during freezing, due to different concentrations of CHT. It can be concluded that pore size increased with increasing concentration of CHT solution, whereas the interconnectivity decreased. Moreover, these values can be influenced by the neutralization method, which in turn affected the morphological features of the scaffolds. Furthermore, the morphological changes observed for the samples with higher concentrations may be related to the partial dissolution and consequent loss of structure during

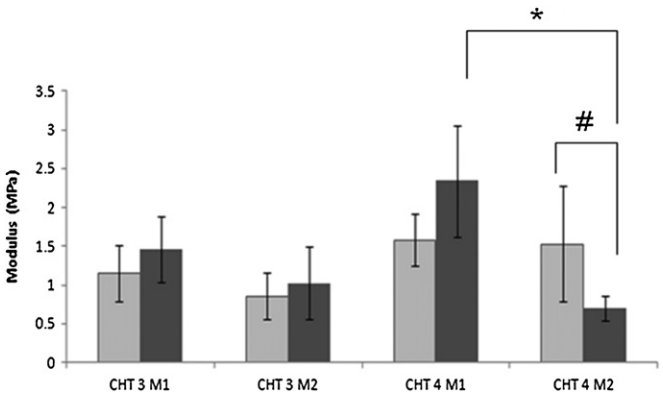

Figure 5. Young's modulus determined from a compression test (dry conditions) to investigate the mechanical properties of porous structures prepared with (■) CHTC and (■) CHTS, (*) represents significant differences between scaffold formulations for the same type of chitosan ($p < 0.05$); (#) represents significant differences between scaffolds prepared with different chitosan origins, but neutralized using the same method ($p < 0.05$).

the neutralization process, namely due to higher reactivity and interactions with solvents such as NaOH and ethanol, as reported in the literature [19]. Another factor that cannot be neglected is the effect of the cooling rate and thus the solvent crystal growth rate, which influenced the final pore size and porosity of the resulting structure after freeze-drying. Furthermore, the pore size ranged between 30–90 μm, which is known to be adequate for tissue engineering purposes [5]. As mentioned before, the neutralization method highly influenced the morphological features of the scaffolds, with CHTC4 M2 scaffolds exhibiting higher porosity and smaller interconnectivity (table 3). Thus, a compromise between the concentration of CHT solutions and neutralization methods must be established in order to render porous structures that exhibit pore sizes that allow cells to populate the interior of the scaffold, but also an adequate interconnectivity that really allows cells to successfully proliferate into the entire polymeric structure [20].

The mechanical properties of those porous structures, in particular the compression modulus, were studied under compression tests in dry conditions. The obtained results for the Young's modulus are illustrated in figure 5.

It was observed that all CHT scaffolds were soft, spongy and very flexible, thus presenting a low resistance to compression. In any case, CHTS scaffolds seem to have better mechanical properties (a higher compressive modulus) than the scaffolds prepared with CHTC, although the observed differences were not statistically significant. These differences may be related to the intrinsic properties of CHT, namely the higher DD of CHTS as observed with CHT membranes, and to the mean pore size of the scaffolds. Moreover, it was observed that the neutralization method also influenced the mechanical properties of the scaffolds. This trend was attributed to the fact that scaffolds neutralized with NaOH (M1) exhibited some shrinkage, but no significant change in volume or shape. However, scaffolds neutralized firstly with ethanol (M2) exhibited some distortion and partial dissolution, which resulted in structures with higher porosity, thus decreasing their mechanical properties [20]. Of all the prepared scaffolds, the ones presenting better mechanical properties were the ones prepared with a higher concentration of CHTS. The enhanced properties may be due to the reduced formation of ice crystals during freezing as a consequence of the higher concentration of CHT.

As previously stated for CHT membranes, porous CHT structures to be used as scaffolds in TE approaches are expected to degrade during cell proliferation, being substituted by expressed extracellular matrix, thus successfully attaining tissue regeneration [17]. Thus, it is important to characterize the structural stability of CHT scaffolds to understand their potential for application in tissue engineering strategies, particularly because their use will depend on many factors such as the DD, the crystallinity of CHT and the intra- and intermolecular forces present in the polymer. The structural stability of the developed CHT scaffolds was investigated by weight loss after incubation in PBS in the presence of lysozyme, during different time periods, in order to simulate physiological conditions. Incubation in PBS in the absence of lysozyme was also performed as a control. After 60 days of incubation, no significant differences were observed on the weight loss profile of scaffolds prepared with CHTC or CHTS, nor of the scaffolds prepared from CHT solutions of 3% or 4%. Moreover, no significant differences were observed on the weight loss of scaffolds neutralized by the two proposed methods. To further explore the structural stability and understand its mechanism, additional tests would be needed, including the measurement of molecular weight after incubation and of enzymatic degradation. Based on the weight loss, one can conclude that the structural stability of CHT scaffolds is not significantly affected by the concentration of the solution used to prepare it, nor by the origin of the CHT used, nor even by the neutralization method, within the conditions used in this work.

Besides the physicochemical properties, when considering a material for application in tissue engineering, it is necessary to assess its cytocompatibility under the processing conditions used. From this perspective, the eventual cytotoxicity of the developed CHT scaffolds was evaluated through MTS assay on extracts from those scaffolds, after being in contact with a mouse fibroblast-like cell line, L929 cells, during 24, 48 and 72 h, following established protocols [24]. The obtained results, shown in figure 6, demonstrate that the developed scaffolds were not cytotoxic and presented cell activity equivalent to that observed with negative controls. Moreover, it is important to highlight that these results show that non-cytotoxic behaviour is independent of the type of CHT used, of the concentration of CHT solution, as well as of the method of neutralization employed. These observations are in accordance to the findings obtained by Chatelet *et al* [25], where CHT was cytocompatibly independent of the DD.

4. Conclusions

Chitin has been successfully isolated from the endoskeletons of the giant squid *Dosidicus gigas* and further converted into CHT, with a high DD (91%). Moreover, highly deacetylated

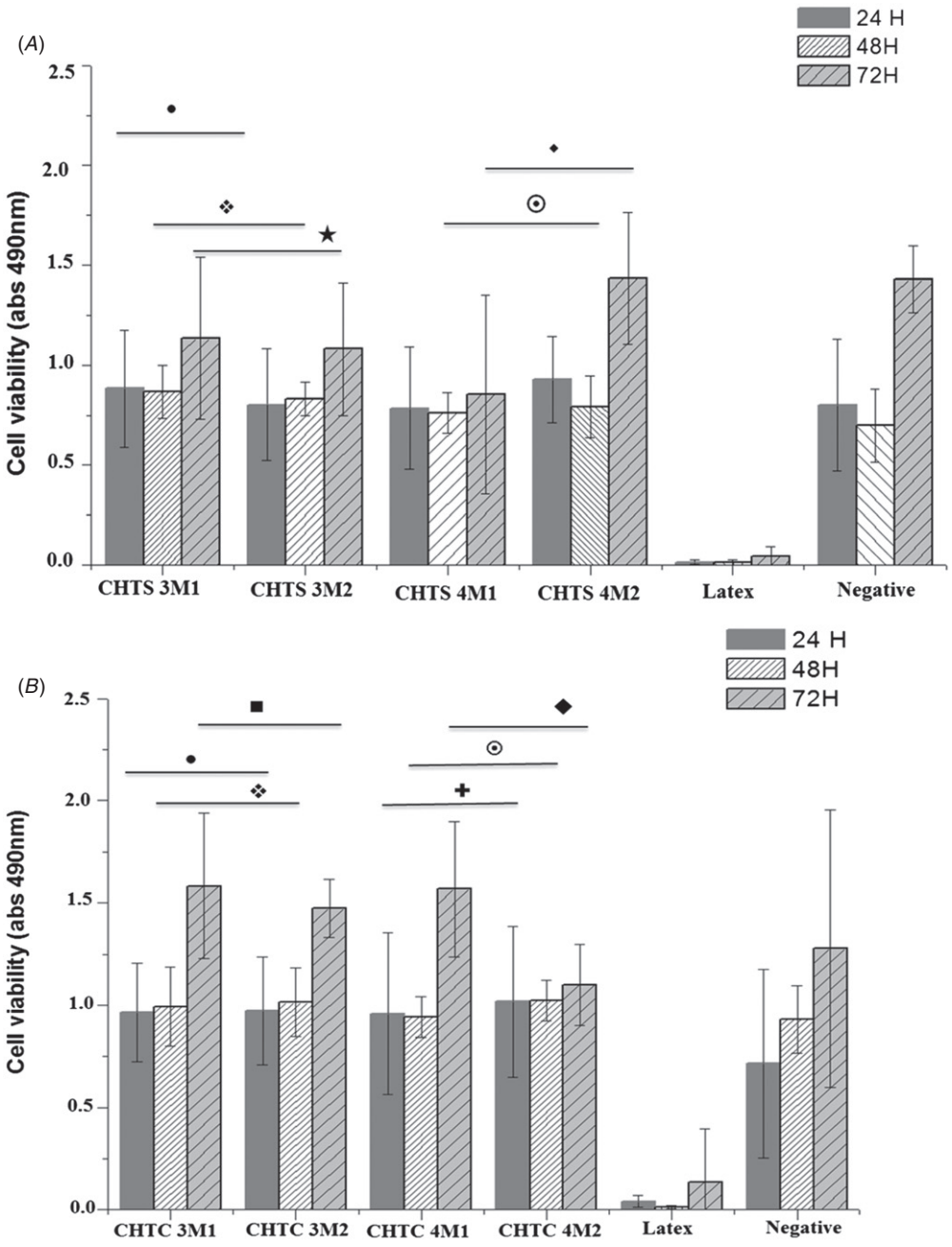

Figure 6. Cytotoxicity assessment with MTS tests on extracts from scaffolds prepared with (*A*) squid chitosan (CHTS) and (*B*) commercial chitosan (CHTC). (❖, ■, ♦, □, ⊙,) represent significant differences between scaffolds for the same types of chitosan for different types of neutralization and the same time of cytotoxicity test ($p < 0.05$).

CHT seems to be easily produced from squid endoskeletons than from crustacean exoskeletons.

With the aim to assess its potential for application in tissue engineering, the produced CHTS was processed into membranes and scaffolds which exhibited equivalent or better properties than the equivalent systems prepared with CHTC. In particular, CHTS systems have similar structural stability and morphological features but better mechanical properties. Regarding cytocompatibility, all produced structures revealed to be non-cytotoxic under the studied conditions. In addition, a new method for the neutralization of CHT structures was proposed, which also leads to higher pore sizes. Thus, a new marine source for the production of a biomedically relevant polymer is presented, reinforcing the potential of marine resources for the biomedical field. Moreover, as the endoskeletons of the giant squid used in this work are obtained from the discards of commercial squid processing, this work has the additional environmental and economical advantage of representing a strategy for the valorization of marine origin residues.

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