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Processing and characterization of 3D dense chitosan pieces, for orthopedic applications, by adding plasticizers.

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Abstract

In this work, plasticizer agents were incorporated in a chitosan based formulation, as a strategy to improve the fragile structure of chitosan based-materials. Three different plasticizers: ethylene glycol, glycerol and sorbitol, were blended with chitosan to prepare 3D dense chitosan specimens. The properties of the obtained structures were assessed for mechanical, microstructural, physical and biocompatibility behavior. The results obtained revealed that from the different specimens prepared, the blend of chitosan with glycerol has superior mechanical properties and good biological behavior, making this chitosan based formulation a good candidate to improve robust chitosan structures for the construction of bioabsorbable orthopedic implants.

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Keywords: chitosan, bioabsorbable implants, plasticizers, mechanical properties, citotocicity

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1. Introduction

Biomaterials have been used extensively for the development of different medical devices. Among those materials, bioabsorbable polymers have attracted special attention since they are capable to decompose gradually inside the human body [1]. Several types of non-bioabsorbable polymers (e.g. ultra-high molecular weight polyethylene, poly(ether-ether-ketone)) or metal components (e.g. stainless steel, cobalt chrome and titanium alloys) have also been used in biomedical applications. However, the bioabsorbable materials offer a better biological interaction with damaged tissues and closely restore the native state, as the mechanical stresses are transferred gradually from the implant to the healing tissue over material degradation [1,2]. In addition, the bioabsorbable polymers eliminate the problems that are associated with the metal fixation devices, such as growth disturbances and hypersensitivity reactions [3].

The bioabsorbable polymers can either be synthetic or natural. The synthetic polymers can be tailored for a wider range of properties and offer higher consistence concerning lot-to-lot uniformity than polymers from natural sources [4]. However, such synthetic materials arguably present problems related with chronic inflammatory reaction and toxicity which can be reduced or even eliminated using natural polymers [3]. Natural polymers are usually macromolecules which the biological environment might recognize and deal with them metabolically, by human enzymes, additionally, depending of the compound, they can be moderately immunogenic [5].

Chitosan is a natural polymer derived from chitin, one of the most abundant natural polymers [6], which is easily obtained from shells of crustaceans (e.g crab and shrimp), cell walls of fungi and cuticles of insects [7]. Chitosan high availability allows envisaging cost effective approaches to commercialization of the final products. The use of chitosan for biomedical applications has been recognized as being of high potential among the medical and pharmaceutical communities. Chitosan reported properties includes good biocompatibility and biodegradability, as well as analgesic, antitumor, haemostatic, antimicrobian and antioxidant properties [8,9]. Moreover, it was also reported that chitosan promote bone formation since it can be shaped into structures and geometries suitable for cell ingrowth and osteoconduction [10].

The process of obtaining chitin from shells of crustaceans consists in the deproteinization of the raw shell material using a diluted sodium hydroxide (NaOH) solution and decalcification in a dilute hydrochloric acid (HCl) solution. On the other hand, chitosan is a derivate product of chitin with a high degree of deacetylation which results from the reaction with an alkali solution (40%-50% NaOH) at elevated temperatures for extended exposures [7,11].

Chitosan is a linear polysaccharide, composed of glucosamine and *N*-acetyl glucosamine linked in a β(1–4) bond. The degree of deacetylation is established as the ratio between glucosamine/N-acetyl glucosamine, which can vary from 50% to 95%. Such polysaccharide is denominated chitin when has a degree of deacetylation lower than 50% [7,11].

Despite being a promising material as a temporary scaffold for bone fractures, currently 3D dense chitosan-based specimens are not easily manufactured and shaped, since their decomposition temperature is lower than their melting temperature [12]. Therefore, heat based processes cannot be used to shape chitosan. Moreover the fragile structure of chitosan also makes extremely challenge to produce 3D dense specimens of different shapes through mechanical processes. Previous reports described that the elasticity of chitosan specimens can be improved and brittleness reduced with the addition of plasticizers on chitosan based materials formulation. Identified plasticizers include polyethylene glycol, glycerol, sorbitol, xylitol, ethylene glycol, propylene glycol [13–15]. Several studies report improvement of the mechanical properties of chitosan films exploring the use of plasticizers, such as polyols (glycerol, sorbitol and xylitol) which interfere with chain-to-chain hydrogen bonding [13,15]. Nevertheless, the use of similar strategy to improve chitosan processability into 3D structures is limited to scarce reports [16].

In this work, three different plasticizer agents - glycerol, sorbitol and ethylene glycol - were added to chitosan and specimens were tested in terms of mechanical, microstructural, physical stability and biocompatibility in order to evaluate the best one for orthopedic applications.

2. Materials and Methods

Chitosan powder was provided by Altakitin S.A. The molecular weight of chitosan was 475 kDa and its deacetylation degree was 92%. The glacial acetic acid was purchased from Carlo Erba Reagents as well as the three plasticizers used: glycerol, the sorbitol and the ethylene glycol. The sodium hydroxide (NaOH) solution (50% w/v) was purchased from Panreac Quimica S.L.U.

2.1. Production of the 3D dense chitosan pieces

The production of the 3D dense chitosan specimens followed the method described by Oliveira *et al.* [16]. In this sense, 3% (w/v) of chitosan was dissolved in 2% (v/v) of acetic acid and 10% (w/w) of the plasticizer (ethylene glycol, glycerol and sorbitol). After the complete dissolution of chitosan, the solution was poured into rectangular moulds and left to rest until all air bubbles disappeared. The moulds were then frozen and subsequently introduced in a NaOH solution, for their precipitation. The resulted specimens were then intensively washed, in order to reach a neutral pH, and then dried at 40ºC. The production process of 3D dense chitosan structures finished with the machination of the desired final shape with a milling machine.

2.2. Mechanical analysis

The 3D dense chitosan specimens were machined to the appropriate shape and dimensions for the compression tests. Triplicates of each condition were tested. The compression tests were conducted following the ASTM standard D695 – 10, using an Instron Testing Machine (model 5566) with a load cell of 10 kN and a loading rate of 1.5 mm/min. The results were processed using the Bluehill®2 Materials Testing Software.

The Compressive Modulus of Elasticity (Young's Modulus) was calculated by the slope of the initial linear portion of the stress-strain curve [17].

The Compressive Strength, i.e. the maximum compressive stress (compressive load per unit area of minimum original cross section carried by the test specimen at any given moment) supported by a test specimen, before a first crack is detected, was also calculated [17].

2.3. Microstructural analysis

Cross sections of the specimens were cut and analyzed in a scanning electron microscope (SEM) using a FEG-SEM equipment with an energy of 10 kV, model JSM-7001F from JEOL. Different photographs of the produced specimens at different magnifications were taken in order to assess the overall topography of those specimens.

2.4. Physical analysis

The density of the chitosan powder was determined by Gas Picnometry. The system used was AccuPyc 1330 Pycnometer, connected to a helium system under pressure (200 bar). The approximate theoretical density of the raw material, used to construct the chitosan specimens, was quantified according to the Equation 1.

$$\boldsymbol{\rho_R = F_C \times \rho_C + F_D \times \rho_D} \quad (1)$$

In this case, ρ_R is the theoretical density of the raw material (i.e. blends of chitosan with plasticizer), F_C is the mass fraction of the chitosan in the final specimen, ρ_C is the density of the chitosan, F_D is the mass fraction of the plasticizer in the final specimen and ρ_D is the density of such plasticizer.

The density of the constructed specimens was determined by the Archimedes Principle. The specimen was weighted (w_A) and then submersed in deionized water. The weight of the specimen in the water was also recorded (w_B). The density of the specimen (ρ_E) was then calculated according to the Equation 2:

$$\rho_E = \frac{w_A}{w_A - w_B} \times \rho_{water} \quad (2)$$

The porosity of the specimens was determined according to the Equation 3. To evaluate the density and porosity of the pieces, triplicates of each condition were tested.

$$Porosity(\%) = \left(1 - \frac{\rho_E}{\rho_R}\right) \times 100 \quad (3)$$

2.5. Biological analysis

An in vitro cytotoxicity assessment of the chitosan specimens was performed. Extract cytotoxicity assay and direct contact cytotoxicity assay were performed according to the international standard ISO 10993-5:2009(E) for medical devices.

Triplicates of each type of chitosan specimen were sterilized with ethanol (70%, v/v) followed by UV exposure (overnight).

In the extract assay, triplicates were submersed in 90% (v/v) Dulbecco's Modified Iscove's Medium (IMDM, GIBCO®) supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Life Technologies) and left in the incubator (37ºC, 21% O_2, 5% CO_2,) for 24h. At the same time, mouse fibroblasts L929 were seeded in 12-well plates in order to achieve 80% of confluence, and were also left in the incubator. After the pre-determined incubation time, the medium in contact with the cells was discarded and the leachings were added to the cells seeded in the well plates. Fresh medium was used as negative control and a piece of latex glove was used as positive control. The well plates were again incubated at the same conditions, for the same period of time. The cell viability was then quantified following the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) protocol. The relative quantification of cell viability was normalized to the negative control. .

To perform the direct contact assay, L929 cells were seeded in well plates as previously described for the extract assay. Triplicates of each type of chitosan specimen were placed on the top of the cell monolayer and incubated in the same conditions and period of time. Cell morphology and viability in contact with materials were then analyzed with the optical microscope Leica DMI3000B.

3. Results and Discussion

The production process described previously, in section 2.1., was effective in the construction of 3D dense chitosan pieces, as shown in Fig.1. The machining process by milling was more difficult for the pieces that had ethylene glycol in their constitution, since they presented higher brittleness and therefore they can easily break during such machining process.

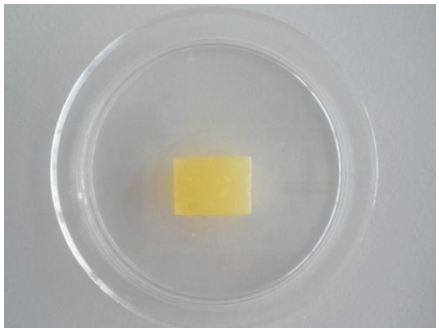

Fig. 1. Example of a 3D dense chitosan piece.

The SEM images of the produced specimens can be observed in Fig. 2 (a)-(c). Each figure represents a different blend of chitosan with a plasticizer, at a 400×magnification.

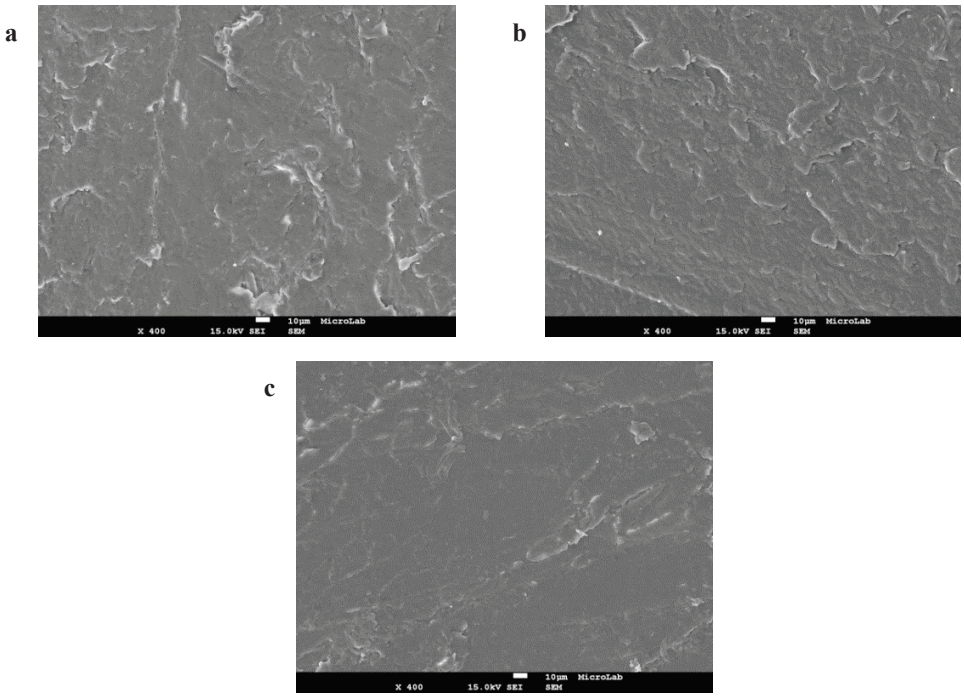

Fig. 2. SEM images of 3D dense chitosan specimens produced by blending chitosan with (a) ethylene glycol; (b) glycerol; (c) sorbitol.

The SEM images revealed that the specimens are mostly dense and do not have perceptible pores at the microscale. The density and the porosity of the specimens were quantified through the Archimedes Principle, considering a chitosan density, determined by Gas Picnometry, of 1,593 g/cm^3, and assuming no material chitosan losses in the production process. The plasticizers had densities of 1,113g/cm^3 for ethylene glycol, 1,262 g/cm^3 for glycerol and 1,285 g/cm^3 for sorbitol as indicated by their manufacturers. The values of density and porosity calculated for the different specimens are shown in Table 1, with respective standard deviations.

Table 1.Density and porosity of the 3D dense chitosan specimens produced by blending chitosan with plasticizers.

Type of blend	Density (g/cm^3)	Porosity (%)
Chitosan + ethylene glycol	1,417 ± 0,008	8,752 ± 1,149
Chitosan + glycerol	1,414 ± 0,005	9,344 ± 0,320
Chitosan + sorbitol	1,413 ± 0,020	9,573 ± 1,280

The results of the compression tests on the three different chitosan blends are shown in Fig. 3. These results are presented as the mean of Young's Modulus and the mean of Compressive Strength, with respective standard deviations.

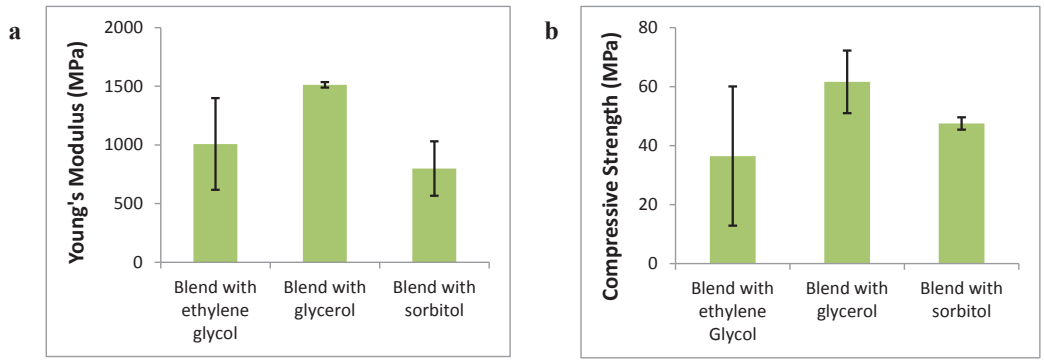

Fig. 3. Mechanical results after compression tests (a) Young's Modulus; (b) Compressive Strength.

As it can be observed in Fig. 3, the blend of chitosan with glycerol presents a higher Young's Modulus as well as a higher Compressive Strength. This reveals that glycerol is a more effective plasticizer agent. According to the literature, a strong hydrogen bonding results from the interaction of chitosan with glycerol, which promotes a better interaction between filler and matrix, facilitating the stress transfer to the reinforcement phase and improving final material mechanical properties [18].

The higher standard deviation associated to the mechanical properties of the blend of chitosan with ethylene glycol is related with the greatest difficulty in machining such pieces, resulting in local fractures and material imperfections due to the mechanical stresses applied during this mechanical process.

The results of the in vitro cytotoxicity tests are shown in Fig. 4 and Fig. 5.

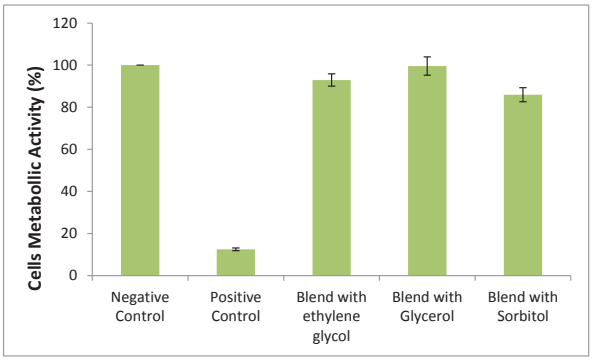

Fig. 4. Results of the cytotoxic test by extract dilution.

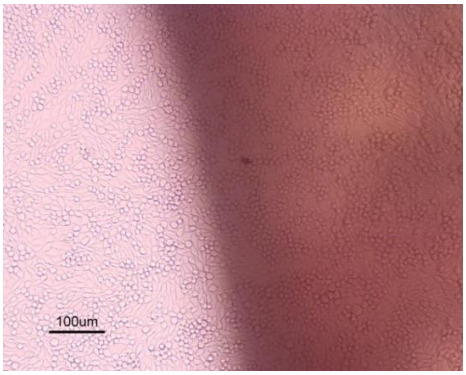

Fig. 5. Example of a cytotoxic test by direct contact for a specimen produced by blending chitosan with sorbitol.

Fig. 4 shows that the metabolic activity of the cells in the presence of lixiviates of the specimens is higher than 80%, reaching the values of the negative control for lixiviates of specimens prepared from not released, for the specimens produced by adding glycerol. These results point outs that significant cytotoxic substances had not been released in the medium during the extract assay. Fig. 5 resumes what was observed in the direct contact assay, for all the specimens, i.e. that the cells grew around and on the surface of the specimens, without any morphologic disorder. These observations reinforce that the blends of chitosan with ethylene glycol, glycerol and sorbitol are not cytotoxic to the cells.

4. Conclusions and Future Directions

From the 3D dense specimens, obtained by blending chitosan with three different plasticizers (ethylene glycol, glycerol and sorbitol), specimens made with glycerol present superior mechanical properties (superior Young's Modulus and Compressive Strength). The physical (porosity less than 10%) and biological (no cytotoxic effect) properties assessed, also suggests that the use of chitosan with the tested plasticizers are good options for development of 3D dense chitosan specimens, which the use the authors envisage for orthopaedic applications. However, the type of plasticizer and its concentration can be optimized and assessed experimentally.

The SEM images and the results of the porosity tests revealed that the produced specimens are dense and do not have perceptible pores at the microscale. However, the porosity results may be lower since they were calculated assuming no losses of chitosan. To obtain more accurate porosity quantification, the final composition of the specimens should be quantified by spectroscopy (e.g. RMN spectroscopy, IR spectroscopy).

The mechanical results revealed higher standard deviations in the case of the chitosan blend with ethylene glycol mainly, due to the higher difficulties in machining such pieces. However, lower standard deviations should be obtained by increasing the number of samples tested to compression.

The results of the biological tests confirmed that the constructed specimens are not cytotoxic to the cells, showing a maximum metabolic activity in the case of the chitosan blend with glycerol.

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