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Bioresorption mechanisms of chitosan physical hydrogels: A scanning electron microscopy study

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ABSTRACT

Tissue-engineered biodegradable medical devices are widely studied and systems must present suitable balance between versatility and elaboration simplicity. In this work, we aim at illustrating that such equilibrium can be found by processing chitosan physical hydrogels without external cross-linker. Chitosan concentration, degree of acetylation, solvent composition, and neutralization route were modulated in order to obtain hydrogels exhibiting different physico-chemical properties. The resulting in vivo biological response was investigated by scanning electron microscopy. "Soft" hydrogels were obtained from chitosan of high degree of acetylation (35%) and by the neutralization with gaseous ammonia of a chitosan acetate aqueous solutions presenting low polymer concentration (Cp = 1.6% w/w). "Harder" hydrogels were obtained from chitosan with lower degree of acetylation (5%) and after neutralization in sodium hydroxide bath (1 M) of hydro-alcoholic chitosan solutions (50/50 w/w water/1,2-propanediol) with a polymer concentration of 2.5% w/w. Soft and hard hydrogels exhibited bioresorption times from below 10 days to higher than 60 days, respectively. We also evidenced that cell colonization and neo-vascularization mechanisms depend on the hydrogel-aggregated structure that is controlled by elaboration conditions and possibly in relation with mechanical properties. Specific processing conditions induced micron-range capillary formation, which can be assimilated to colonization channels, also acting on the resorption scenario.

1. Introduction

Scaffold material development represents a large field of research in tissue engineering. The scaffold concept has evolved since last decades and refers to cell-seeded or cell-free, single- or multi-material based devices that are likely to be bioresorbed and invaded by cells. Among the different parameters that are essential for the functional properties of scaffolds (e.g. biodegradation, absence of toxicity, cell function compatibility) [1], the time scale of the cellular colonization and bioresorption of the implant is a key parameter to control in order to address a large range of biological applications [2,3]. A complementary strategy in the design of materials for tissue engineering is the 'decoy material' concept [4]. It consists in the partial replication of the physical and chemical structure of living media, so as to induce their favorable response. In this view, natural polysaccharides with a chemical structure partially found in extracellular matrices are of particular interest, such as chitosan [4].

Chitosan is constituted by D-glucosamine and N-acetyl-D-glucosamine residues with β(1–>4) glycosidic linkages, thus resulting in a partial analog of hyaluronic acid. In addition, several studies have evidenced hydrogels as the most suitable physical state for such approach [5,6] since natural tissues are complex hydrogels themselves. Chitosan [7–9] and other polysaccharides such as alginates [10,11] or hyaluronic acid [12,13] are widely used for the preparation of hydrogel scaffolds with high water content (up to 99%). Such biomaterials can be modified with physical or chemical cross-linkers in order to tune their mechanical behavior [14,15]. Coating with other bioactive polymers is also assessable to modify their surface properties (e.g. heparin to reduce the thrombosis effect [16]). A specific bio-functionalization can be obtained by pre-cellularization of implants to improve their biological responses [17]. As a result, the most sophisticated scaffold materials are multi-component systems involving the utilization of possibly toxic, reactive molecules as cross-linkers, which require a complex device elaboration and possibly a difficult acceptance of commercial and marked devices in the regulatory context. For all those reasons, the approach followed in this study involves in a first step the development of uncellularized and tuneable chitosan-based materials, processing in

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the absence of chemical modification or complex formulation strategies but acting mainly on physico-chemical parameters.

The chitosan polymer family includes all the deacetylated derivatives of chitin that stay soluble in acidic aqueous solutions (ex: acetic or hydrochloride solutions). Chitosans are usually characterized by their molecular mass distributions, their degree of acetylation (DA, i.e. the global molar fraction of acetylated residues) and the intramolecular repartition of N-acetyl D-glucosamine (GlcNAc) and D-glucosamine (GlcN) residues. The protonation of amine groups (the pKa ranging between 6.5 and 7 with increasing DA values [18]) is responsible for the polymer hydrophilicity in aqueous solution. Conversely, neutralization of chitosan solutions by increasing the pH above the pKa value can lead to physical hydrogels if the kinetics of gelation is fast enough to avoid the formation of a (disentangled) precipitate [19].

Physical chitosan hydrogels present exceptional biological properties (biocompatibility, biodegradability and bioactivity) [20,21]. Moreover, because of their versatile elaboration process, many parameters such as the polymer concentration, DA, solvent nature of the initial chitosan solution (water or water/1,2 propanediol mixtures) and neutralization procedure (i.e. neutralization with gaseous ammonia or sodium hydroxide baths) can be modulated in order to vary the physico-chemical properties of the resulting hydrogels. Thereby, this work aims at developing physical chitosan hydrogels presenting a set of biological properties depending on the elaboration conditions. To this end, different hydrogels were implanted in Wistar rats as subcutaneous implants and the explants were further investigated in 3D by scanning electron microscopy in low vacuum mode.

2. Material and methods

2.1. Materials

The initial highly deacetylated chitosan, produced from squid pens with high molecular weight, was supplied by Mahtani Chitosan Pvt. Ltd. (India, Mahtani indexes 114, batch N°S3, January 2011). 1,2-propanediol (purity of >99.5%) as well as sodium hydroxide pellets, ammonium hydroxide solution at 28–30% (w/w) and acetic acid were purchased from Acros organics or Sigma Aldrich.

2.2. Chitosan purification

In order to obtain a high-purity material, chitosan was dissolved at 0.5% (w/v) in an aqueous acetic acid solution, by the addition of the necessary amount of acid to achieve the stoichiometric protonation of the $-NH_2$ sites. After complete dissolution, the chitosan solution was sequentially filtered through the Millipore membranes with pore sizes of 3, 1.2, 0.8 and 0.45 μm in order to eliminate the insoluble polymer residual fraction. Then, dilute ammonia was added to the filtered chitosan solution to fully precipitate the polymer. Finally, the precipitate was repeatedly rinsed with distilled deionized water until a neutral pH was achieved. Then it was centrifuged and lyophilized.

2.3. N-acetylation of chitosan

The N-acetylation of chitosan was obtained in a water–1,2-propanediol mixture with acetic anhydride as the reagent. Thus, an aqueous acetic acid solution of chitosan was prepared at a concentration of about 1% (w/w): chitosan was dissolved in deionized water containing the amount of acetic acid necessary to achieve the stoichiometric protonation of the NH_2 sites. Then, 1,2-propanediol was added to achieve a final polymer concentration of 0.5% (w/w), with equal amounts of water and alcohol (50%/50% (w/w)). A solution of pure and fresh acetic anhydride in 1,2-propanediol was slowly added under strong stirring. The amount of acetic anhydride corresponded to the stoichiometric amount necessary to achieve a given degree of acetylation (5% and 35% in this study). The medium was left to stand for 3 h. The obtained chitosan was precipitated with aqueous ammonia (28% (w/w)). The polymer was washed in deionized water and lyophilized as previously described.

2.4. ^1H nuclear magnetic resonance spectroscopy

The degree of acetylation (DA) of chitosan was calculated from ^1H nuclear magnetic resonance spectroscopy [22]. 10 mg of purified chitosan was dissolved in 1 mL of D_2O containing 0.06 mM of HCl. Spectra were recorded on a Bruker ALS 300 spectrometer (300 MHz) at 25 °C. The DA was deduced from the ratio of the area of the peaks of the methyl protons of the N-acetylglucosamine residues to that of all of the H2 to H6′ protons of both glucosamine and N-acetylglucosamine residues [22].

2.5. Size exclusion chromatography coupled with multiangle laser light scattering

The weight–average molecular weight of chitosan and polydispersity index were determined by a size exclusion chromatograph (SEC) coupled online with a differential refractometer (Waters R410, from Waters-Millipore) and a multiangle laser-light scattering detector operating at 632.8 nm (Wyatt Dawn DSP). The refraction index increment dn/dc depends on the DA as determined in previous studies [18]. For the chitosan samples used in this study, dn/dc is close to 0.198 mL/mg. A 0.15 M ammonium acetate/0.2 M acetic acid buffer (pH = 4.5) was used as eluent at a flow rate of 0.5 mL/min on Tosoh TSK PW 2500 and TSK PW 6000 columns. The polymer solutions were prepared by dissolving 0.5 mg of polymer in 1 mL of buffer and then refiltered through a Millipore membrane with a pore size of 0.45 μm before an injection of 100 μL. The molecular mass of chitosan was kept constant for all the study and close to 600,000 $g \cdot mol^{-1}$.

2.6. Preparation of physical chitosan hydrogels

Physical chitosan hydrogels were prepared according to two different processing routes: (i) gelation of aqueous chitosan solutions, and (ii) gelation of 1,2-propanediol/water hydro-alcoholic chitosan solutions presenting increased viscosity [19,23–25]. In both routes, solutions were first obtained by dispersing purified chitosan lyophilizates into water, and then acetic acid was added to achieve the stoichiometric protonation of $-NH_2$ sites. In the aqueous solution route, chitosan and water amounts were settled to give the final needed concentration of 1.6% (w/w). In the hydro alcoholic solution route, the quantity of water was halved, and the same mass of 1,2-propanediol was added (after the complete dissolution of chitosan in the aqueous acetic acid solution) to reach the final concentration of 2.5% (w/w). The resulting solutions were centrifuged for 10 min at 5000 rpm using ProcessMate 5000 centrifuge (Nordson EFD) to remove air bubbles. Solutions were then neutralized by direct contact with sodium hydroxide bath at a concentration of 1 M for 1 h, or under ammonia vapors for 15 h (vapors generated by 100 mL of 1 M NH_4OH solution placed into a 5 liter desiccator). The resulting hydrogels were washed into deionized water until neutral pH and complete elimination of the alcohol (for hydro-alcoholic solution route hydrogels). Specimens were stored into

Table 1
Polymer concentration (Cp), degree of acetylation (DA), solubilization route (SR) and neutralization route (NR) of chitosan physical hydrogels used in this study.

Sample	Cp (% w/w)	DA (%)	SR	NR
H1	1.6	5	Aqueous	NH_4OH[a]
H2	1.6	20	Aqueous	NH_4OH[a]
H3	1.6	35	Aqueous	NH_4OH[a]
H4	1.6	5	Aqueous	NaOH
H5	2.5	5	Hydro-alcoholic	NaOH

[a] Vapours.