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Fine microstructure of processed chitosan nanofibril networks preserving directional packing and high molecular weight

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ABSTRACT

Crystalline chitosan nanofibril networks were prepared, preserving the native structural packing and the polymer high molecular weight. The fine microstructure of the nanomaterial, obtained by mild hydrolysis of chitosan (CHI), was characterized by using synchrotron small- and wide-angle X-ray scattering (SAXS and WAXS), transmission electron microscopy (TEM) and electron diffraction. Hydrolysis of chitosan yielded a network of crystalline nanofibrils, containing both allomorphs of chitosan: hydrated and anhydrous. The comparison of WAXS data in transmission and reflection mode revealed the preferential orientation of the CHI crystals when subjected to mechanical compression constrains. The results are in agreement with the existence of a network nanostructure containing fiber-like crystals with the principal axis parallel to the polymer chain axis. The evolution of the CHI allomorphic composition with temperature was studied to further elucidate the mechanism of structural transitions occurring during CHI nanofibril network processing.

1. Introduction

Nanocrystals from natural polymers are attracting an increasing interest for their potential use as nanofillers in nanocomposites. The valorization of biomass in the form of crystalline nanofibers constitutes a revolution in materials science. Most of the studies have been devoted to cellulose and chitin nanocrystals (Dufresne, 2012; Eichhorn et al., 2010; Favier, Chanzy, & Cavaillé, 1995; Lin, Huang, & Dufresne, 2012; Osorio-Madrazo et al., 2012). Many investigations describe the preparation of cellulose (Dufresne, 2012; Elazzouzi-Hafraoui et al., 2007; Lin et al., 2012) and chitin (Fan, Fukuzumi, Saito, & Isogai, 2012; Gopalan Nair & Dufresne, 2003) whisker nanocrystals by heterogeneous acid hydrolysis.

Chitosan (CHI) is a linear polysaccharide, copolymer of (1→4)-linked units of 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glucopyranose, mainly produced by deacetylation of chitin. The main chitin source is the cuticle of crustaceans, in which chitin is organized into fiber bundles (Erko et al., 2013; Fabritius et al., 2012). Besides its biodegradability and biocompatibility, CHI presents the rare property of bioactivity. A number of *in vitro* and *in vivo* studies have highlighted its biological properties (Gorzelanny, Pöppelmann, Pappelbaum, Moerschbacher, & Schneider, 2010; Ladet, Tahiri, Montembault, Domard, & Corvol, 2011; Mathews, Gupta, Bhonde, & Totey, 2011; Montembault et al., 2006). CHI nanofibrils can be used as reinforcement of biomaterials for technological, biotechnological and biomedical applications. Nevertheless, the preparation of highly crystalline CHI nanofibrils that preserve the native directional structural packing and macromolecular structure is challenging. We previously reported on acid hydrolysis as a method to increase crystallinity of CHI substrates (Osorio-Madrazo et al., 2010; Osorio-Madrazo et al., 2011). The deacetylation of chitin nanowhiskers has been proposed to produce CHI nanofibers. Nevertheless, it yielded neither distinguishable needlelike CHI nanowhiskers nor a network of crystalline nanofibrils (Lertwattanaseri, Ichikawa, Mizoguchi, Tanaka, & Chirachanchai, 2009; Phongying, Aiba, & Chirachanchai, 2007; Rinki et al., 2009; Wijesena et al., 2015). Instead, the deacetylation of chitin whiskers yields a CHI network with a significant loss

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of crystallinity. By deacetylating chitin whiskers in $NaOH/NaBH_4$ (Watthanaphanit, Supaphol, Tamura, Tokura, & Rujiravanit, 2010), CHI nanoparticles resembling whiskers were obtained but with a very low aspect ratio (L/d: ~4.8), significantly lower than those reported for chitin and cellulose whiskers (L/d: 20–130) (Elazzouzi-Hafraoui et al., 2007; Osorio-Madrazo et al., 2012). Moreover, the degree of N-acetylation (*DA*) of the copolymer was about 50%. Thus, the obtained particles still should be referred to as chitin whiskers instead of chitosan. For the most common definition of CHI – a copolymer soluble in weak acidic aqueous media – CHI obtained by heterogeneous deacetylation should have a *DA* below 40%. However, it has been disclosed that prolonged deacetylation treatment of chitin whiskers compromises the integrity of the fibrillar morphology (Phongying et al., 2007; Watthanaphanit et al., 2010). The electrospinning of CHI solutions was proposed to produce CHI nanofibers (Haider et al., 2013; Martinova & Lubasova, 2008). However, a high crystallinity could not be achieved. The preparation of CHI nanofibrils, preserving both the native structural packing and polymer high molecular weight has not yet been reported. Most of the studies on CHI crystals have been performed on previously solubilized oligomers (*e.g. DP* ~35), in which the native packing was destroyed (Belamie, Domard, Chanzy, & Giraud-Guille, 1999; Cartier, Domard, & Chanzy, 1990; Cartier, Mazeau, Domard, & Chanzy, 1992). CHI polymer chains preferentially organize into lamellar single crystals (Belamie et al., 1999) and the preparation of fibrillar nanocrystals remains a challenge (Belamie, Domard, & Giraud-Guille, 1997).

In addition to the CHI intrinsic bioactivity, highly crystalline nanofibrils should significantly enhance the mechanical performance of CHI nanofibril-reinforced composites. In the present work, we describe the preparation of CHI nanofibrils networks by mild hydrolysis of semicrystalline CHI flakes. The morphology, ultrastructure and crystallinity of the treated CHI samples were characterized using small- and wide-angle synchrotron X-ray scattering (SAXS and WAXS), transmission electron microscopy (TEM) and electron diffraction.

2. Materials and methods

2.1. Molecular weight

Chitosan molecular weights (M_w and M_n) were determined by size-exclusion chromatography (SEC). About 0.1% (w/v) CHI solutions were prepared in an AcOH (0.2 M)/$AcONH_4$ (0.15 M) buffer (pH = 4.5) used as eluent; then, filtered through acetate cellulose membranes (Millipore, 0.22 μm). SEC equipment was composed of an IsoChrom LC pump (Spectra-Physics) connected to a Protein Pack 200 SW (WATERS) column and a TSK gel G6000 PWXL (TOSOH BioScience). A multi-angle laser light scattering (MALLS) detector DAWN DSP (Wyatt) was coupled on line with a WATERS 410 differential refractometer.

2.2. Degree of N-acetylation

10 mg of CHI were dissolved in 1 mL of D_2O acidified with 5 μL of concentrated HCl (12 M) and analyzed by ^1H NMR spectroscopy on a Bruker ALS 300 spectrometer (300 MHz for 1H) at 298 K. *DA* was calculated from the ratio of the methyl proton signal of N-acetylglucosamine (GlcNAc) residues to the whole H2–H6 proton signals (Hirai, Odani, & Nakajima, 1991).

2.3. Wide- and small-angle X-ray scattering (WAXS and SAXS)

WAXS patterns in reflection mode were collected with a Siemens D 500 diffractometer (CuKα, λ = 0.1542 nm) operating at 35 kV and 30 mA. The CHI crystallinity index *CrI* was determined from the ratio of the crystalline peaks contribution to the total area of the diffraction patterns (Osorio-Madrazo et al., 2010). Complementary synchrotron SAXS and WAXS patterns in transmission mode were recorded at the BM02/D2AM beamline at the ESRF, Grenoble (photon energy: 16 keV, λ = 0.7749 Å). Data were collected using a CCD detector (Ropper Scientific). The contribution of the empty cell was subtracted from the scattered data. Silver behenate was used as *q*-range calibration standard.

2.4. Transmission electron microscopy (TEM) and electron diffraction

Chitosan particles suspended in water were dispersed with an Ultra-Turrax homogenizer. Droplets of the dilute dispersion were deposited on carbon-coated grids and allowed to dry. Specimens were observed with Philips CM120 and CM200 microscopes operating at 80 kV. For electron diffraction, suspension droplets were dried on Pelco NetMesh lacy films. Specimens were quench-frozen in liquid nitrogen prior to introduction into the microscope. Diffraction patterns were recorded at low temperature (−175 °C) with the Philips CM200 microscope operated at 200 kV, on Fujifilm imaging plates read with a Fujifilm BAS 1800II bio-imaging system.

2.5. Water content

CHI water content was determined with a Thermogravimetric Analyzer TGA 2950 (Dupont instruments) from the sample weight loss measured between 25 and 200 °C. A temperature ramp of 2 °C min^{-1} up to 200 °C under Helium flow was applied.

2.6. Preparation of chitosan nanofibril networks by mild acid hydrolysis

Chitosan flakes (~5 mm^2) were impregnated with concentrated HCl to perform the solid state hydrolysis. Hydrolysis parameters like the acid concentration, the time and the temperature of the reaction were investigated. HCl concentrations of 3 and 12 M, hydrolysis times from 0 to 48 h, and temperatures of 5; ~22 and 50 °C were considered. A molar ratio of glucosamine (GlcN) units to water (the reactant) equal to 60 resulted to be the optimal as to perform reproducible hydrolysis studies (see supplementary data). The added HCl volume was calculated according to Eq. (S2) (see supplementary data). The hydrolyzates were washed firstly with 1.5 M HCl to eliminate low *DP* (≤15) oligomers, secondly with 1.0 M ammonia and thirdly with distilled water until neutral pH and complete desalination. After filtration, the product was isolated by freeze-drying. Multi-step hydrolyses were also performed, with inter-steps washings as above.

3. Results and discussion

3.1. Characterization of starting chitosan flakes and fully deacetylated chitosans

The starting chitosan ch1 from shrimp shell chitin was supplied by Mahtani Chitosan, India (Batch No. 2224). In the industrial process developed by Mahtani Chitosan, both the chitin extraction and chitosan production were carried out in heterogeneous conditions. Consequently, chitin and chitosan particles were never solubilized during the process, preserving the natural semicrystalline state of native chitin. The initial ch1 characterization yielded M_n of $1.48 \pm 0.07 \times 10^5$ g mol^{-1}, *DA* of 22.7%, water content of 9.5 wt% and *CrI* of 56.7%. To enhance the crystallinity in CHI nanofibrils, a higher ordering of the polymer chains is expected from a homopolymer structure. Thus, the full deacetylation of chitosan was performed: