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Production and characterization of a nanocomposite of highly crystalline nanowhiskers from biologically extracted chitin in enzymatic poly(ε-caprolactone)

Andres Espadín[a], Lenin Tamay De Dios[b], Erika Ruvalcaba[b], Josefina Valadez-García[c], Cristina Velasquillo[b], Ismael Bustos-Jaimes[c], Humberto Vázquez-Torres[a], Miquel Gimeno[d,*], Keiko Shirai[a,*]

[a] Universidad Autonoma Metropolitana, Biotechnology Department, Laboratory of Biopolymers and Pilot Plant of Bioprocessing of Agro-Industrial and Food By-Products, Av. San Rafael Atlixco No. 186, Iztapalapa, 09340, Mexico City, Mexico
[b] Laboratorio de Biotecnología, Instituto Nacional de Rehabilitación, Ciudad de Mexico, Mexico
[c] Depto. de Bioquímica, Facultad de Medicina, Universidad Nacional Autónoma de México, Ciudad de México, 04510, Mexico
[d] Depto. De Alimentos y Biotecnología, Facultad de Química, Universidad Nacional Autónoma de México, Ciudad de México, 04510, Mexico

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ABSTRACT

A nano-composite from biologically obtained chitin nanofillers homogenously dispersed in a poly(ε-caprolactone) matrix was successfully achieved by an ultrasonication-assisted non-toxic and non-aqueous methodology. For this purpose, biological chitin was obtained from lactic acid fermentation of shrimp wastes and converted into chitin whiskers by acidic hydrolysis in a novel process at low temperature (4 °C) that enhanced the distribution and yield. Additionally, the polyester matrix was enzymatically produced in a non-toxic compressed fluid (1,1,1,2-tetrafluoroethane at 25 bar and 65 °C) medium. The homogeneous distribution of the nanofiller in the matrix was corroborated by confocal and atomic force microscopies. Films of the nanocomposite were physicochemically characterized to assess its adequate properties. Additionally, the qualitative viability of human fibroblasts and osteoblasts cells was studied on the produced nanocomposite films showing good biocompatibility.

1. Introduction

The production of nanocomposites (Nc) based on degradable biomaterials has shown uprising interest in the field of nanobiotechnology towards their application in regenerative medicine. Enhanced mechanical and barrier properties in Nc are achieved with nanofiller reinforcements distributed in adequate matrices. Among them, the thermoplastic and semi-crystalline poly-ε-caprolactone (PCL) is desired for tissue engineering owing to its excellent degradability, biocompatibility, and processability (Morin & Dufresne, 2002; Woodruff & Hutmacher, 2010). Additionally, the PCL can also be produced enzymatically in non-toxic and green solvents thereby with the absence of toxic residues from synthesis toward a product with similar characteristics to that from chemical routes (Corr, 2002; García-Arrazola, Gimeno, & Barzana, 2007; Loeker et al., 2004).

In respect to the fillers, these include the use of non-bio degradable inorganic clays, layered double hydroxides, and bioglass, among others (Jo et al., 2009). Nonetheless, the inclusion of nanoparticles of biopolymers enhances the biological activities and biodegradability, which are highly desirable for biomedical applications (Habibi & Dufresne, 2008; Morin & Dufresne, 2002; Zeng, He, Li, & Wang, 2012). In this regard, chitin is pointed out owing to good biocompatibility and biodegradability, as well as its wound healing ability. More importantly, this polysaccharide favors cell adhesion and proliferation (Espadín et al., 2014; Jayakumar, Deepthy, Manzoor, Nair, & Tamura, 2010; Ji, Wolfe, Rodríguez, & Bowlin, 2012; Shahidi & Abuzaytoun, 2005; Synowiecki & Al-Khateeb, 2003). Chitin is composed of β-(1-4)-2-acetamido-2-deoxy-NULL-glucopyranose and β-(1-4)-2-deoxy-NULL-glucopyranose units and naturally found in the form of nanometric (2.5–2.8 nm) fibrils embedded in protein matrices in the exoskeleton of crustaceans. The commercial extractions of chitin from crustacean shells by chemical routes are effective. However, the methodologies

* Corresponding authors.
E-mail addresses: mgimeno@unam.mx (M. Gimeno), smk@xanum.uam.mx, kshiraim@gmail.com (K. Shirai).

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induce depolymerization, loss of other value-added side products and deacetylation in addition to increasing amorphous areas in the final product (Pacheco et al., 2011). Alternatively, previous works showed the advantages of biological approaches for the recovery of chitin by lactic acid fermentation (LAF) to produce high molecular weight chitin with the recovery of protein and pigments and more interestingly, having higher crystallinities than others from chemical routes (Pacheco et al., 2011). This increase in crystallinity has advantages in the biomedical applications of this polysaccharide (Pacheco et al., 2011; Synowiecki & Al-Khateeb, 2003). To produce nanowhiskers of chitin (CHW), also known as chitin nanofibril crystals, the preservation of the crystallinity within biological extraction is crucial, and thereby, the reported methods based on the separation of the amorphous areas become more rapidly hydrolyzed than the crystalline ones under strong acid suspensions. Other approaches include TEMPO-mediated oxidation or ionic liquid treatments (Ji et al., 2012; Mincea, Negrulescu, & Ostafe, 2012; Zeng et al., 2012). Also, Ifuku et al. reported an unusual mechanical approach to obtain high-aspect ratio chitin nanofibrils (Ifuku et al., 2009, 2010).

Nonetheless, for effective Nc production, homogeneous dispersion without aggregation in the matrix is also to be accounted for (Morin & Dufresne, 2002). PCL is insoluble in water, therefore approaches for embedding nanofillers of polysaccharides in this matrix include the use of large amounts of surfactants to the aqueous system (Morin & Dufresne, 2002). The modification of nanoparticle surfaces or linkage of chitin and PCL chains is susceptible to induce changes in the crystals that consequently might affect the Nc properties (Ji et al., 2012; Zeng et al., 2012). Other approaches have been described recently for the preparation of Nc of CHW embedded in PCL matrix with the use of 2,2,2-trifluoroethanol by solvent-cast films or electrospun fiber mats (Ji et al., 2012; Ji, Liang, Shen, & Bowlin, 2014). These authors suggested that the hydrogen-bonding capacity of the 2,2,2-trifluoroethanol and its weak acidity restricts the agglomeration of CHW and also maintain the positive charge (NH_3^+) thereby the inter-particle electrostatic repulsions. However, this fluorinated solvent classified as highly toxic which might restrict potential biomedical applications.

The present study demonstrates that low temperature (4 °C) acid hydrolysis of highly crystalline biologically extracted chitin allows the production of smaller particle sizes than other reported routes (Goodrich & Winter, 2007; Rubentheren, Ward, Chee, & Tang, 2015; Xia et al., 2017). This novel Bio-CHW nanofiller homogenously dispersed in the PCL matrix, which was enzymatically produced by lipase-based biocatalysts in the liquid 1,1,1,2-tetrafluoroethane green-solvent medium. The films of this bio-CHW in the enzyme-mediated PCL proceed by ultrasonication-assisted non-toxic and non-aqueous methodology. The conducted physicochemical characterizations and a qualitative study on cell viabilities of human fibroblast (HF) and osteoblasts (HO) cells are discussed.

2. Materials and methods

2.1. Materials

Cephalothoraces of shrimp (*Litopenaeus vannameii*) were acquired in Mexico City's central seafood market. Chitin was purified following the LAF methodology reported by Pacheco et al. (2011) using *Lactobacillus plantarum* as the fermentative microorganism in a 34% yield on dry weight basis. Chitin characteristics were identically measured as described by Pacheco et al. (2011) to obtain ash content of 1.06 ± 0.196% and protein content of1 ± 0.068%, M_v of 603,700 g/mol, DA of 96.7% and crystallinity index (I_{CR}) of 94.1%. Enzyme-mediated PCL in liquid 1,1,1,2-tetrafluoroethane medium was produced by ring-opening polymerization of epsilon-caprolactone (Sigma-Aldrich, USA) previously distilled over CaH_2 following the method reported by García-Arrazola et al. (2007) in 400 mL high-pressure reactor volume exhaustively dried before the reaction. Immobilized Lipase B from *Candida antarctica* biocatalyst with an enzymatic activity of 6000 U/mg was supplied by C-Lecta (GER). Initial water activity was < 0.16 as measured in hygrometer (Rotronic aw-quick, USA) at 25 °C. The reaction proceeded at 25 bar and 65 °C for 24 h. Reactor contents after depressurization at atmospheric pressure were removed with chloroform, biocatalyst separated by filtration and PCL precipitated in cold methanol (1/10 vol/vol). PCL was obtained as white fibers in 90% yield. PCL characteristics were identically measured as reported by García-Arrazola et al. (2007) to obtain M_n = 37,152 g/mol (PDI = 2.2), T_m = 55.6 °C, TGA = 475 °C and percentage of crystallinity = 54.3%. *N,N*-dimethylacetamide (98% purity) and lithium chloride (LiCl) were supplied by Sigma (USA). HCl, NaOH, and acetone (reagent grade) were supplied by JT Baker (Mexico). Chloroform and ethyl acetate (AcOEt) (HPLC grade) were acquired from J.T Baker (Mexico). Chloroform and ethanol (technical grade, Casa Miyako, Mexico) were used as supplied. Dulbecco's Modified Eagle Medium (DMEM F12), fetal bovine serum (FBS; Gibco, USA), phosphate buffered saline (PBS), penicillin-streptomycin and amphotericin B were supplied by Gibco (USA).

2.2. Production of bio-CHW

Biological chitin was sieved through Tyler mesh No. 6, 12 and 150 with the mesh opening sizes of 3.3, 1.4 and 0.1 mm, respectively. Samples (1 g) were suspended in 15 mL of HCl solution (12 M) at 4 °C for periods of 6, 7 and 8 h. In another experiment, following the reported method by Morin & Dufresne (2002), a sample sieved to 0.1 mm was hydrolyzed at 90 °C with HCl (3 M) during 1.5 h. Then, suspensions were diluted with 30 mL of distilled water and centrifuged at 14,000 rpm for 20 min (4 °C). This procedure was repeated three times removing the water after each centrifugation. Then, a solution of NaOH (7 M) was added at pH 5 and centrifuged. After removing the water, the Bio CHW was collected and dialyzed with deionized in water (18 MΩ cm at 25 °C) for 24 h (water replacement every 8 h) at 5 °C until reach pH 7. Finally, the samples were lyophilized and stored at room temperature until use. The experiments were conducted by triplicates for each condition.

2.3. Production of bio-Nc films

In a typical experiment, bio-CHW (1 g) obtained from the treatment of the 0.1 mm sieved chitin for 8 h at 4 °C was suspended in 10 mL of AcOEt: chloroform in 1:3 (v/v) ratio. The mixture was ultrasonicated for 10 min in a Branson 2510 Sonicator (Bransonic, USA) and allowed to stand for 12 h to precipitate the aggregates, the supernatant containing the suspended non-aggregated nanoparticles was recovered and dried (100 °C), which accounted for 10 wt% of the initially suspended particles. Then, 0.02, 0.04, 0.06, 0.08 and 0.1 g of CHW were placed in flasks with 25 mL of chloroform (HPLC grade) which will correspond to samples PW2, PW4, PW6, PW8, and PW10, respectively. Suspensions were ultra-sonicated at 5 °C in a Sonics VCX 130PB (USA) for 10 min (70% amplitude). Then, PCL (1 g) was dissolved in each suspension and formation of Nc proceeded after solvent evaporation at room temperature under magnetic stirring. Samples were vacuum dried (4×10^{-4} mbar) *before* being sandwiched between glass plates and melted (100 °C) under pressing (4 kg), then, films were easily removed after cooling at 4 °C. Control PCL film sample was identically produced without the presence of CHW.

2.4. Characterization of the materials

Infrared (FT-IR) spectra were acquired on a Perkin-Elmer GxFT-IR Spectrum (UK) with a resolution of 2 cm^{-1} and 32 scans. Samples were analyzed in cesium iodine (Perkin–Elmer USA) discs with the addition of 1 μL of chloroform to form a translucent microfilm. Nanoparticle size distributions were measured by the change in the diffraction index (DI)