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Bio-mimetic composite scaffold from mussel shells, squid pen and crab chitosan for bone tissue engineering

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ABSTRACT

In the present study, chitosan/hydroxyapatite (HA)/β-tircalcium phosphate (β-TCP) composites were produced using squid pen derived chitosan (CHS) and commercial crab derived chitosan (CHC). CHS was prepared from squid pens by alkaline N-deacetylation. HA and β-TCP were extracted from mussel shells using a microwave irradiation method. Two different composites were prepared by incorporating 50% (w/w) HA/(β-TCP) in CHS or CHC followed by lyophilization and cross-linking of composites by tripolyphosphate (TPP). The effect of different freezing temperatures of −20, −80 and −196 °C on the physicochemical characteristics of composites was investigated. A simulated body fluid (SBF) solution was used for preliminary *in vitro* study for 1, 7, 14 and 28 days and the composites were characterized by XRD, FTIR, TGA, SEM, μ-CT and ICP-MS. Porosity, pore size, water uptake; water retention abilities and *in vitro* degradations of the prepared composites were evaluated. The CHS composites were found to have higher porosity (62%) compared to the CHC composites (porosity 42%) and better mechanical properties. The results of this study indicated that composites produced at −20 °C had higher mechanical properties and lower degradation rate compared with −80 °C. Chitosan from the squid pen is an excellent biomaterial candidate for bone tissue engineering applications.

1. Introduction

In recent years, much attention has been paid to marine by-products, scoping their cost-effective processing schemes and their potential for production of high-value products. Natural polymers like chitin, chitosan and calcium phosphate (CaP) compounds can be obtained from waste marine products [1]. Because of their intrinsic properties such as biocompatibility, biodegradation and antimicrobial properties, these natural materials have important biomedical applications [2,3]. In the last two decades, many reports have been published on chitin and chitosan applications in drug delivery, tissue engineering, skin and bone grafting [2,4,5]. Chitosan is a biopolymer consisting of (1,4)-2-amino-2-deoxy-D-glucose units that is obtained by N-deacetylation of chitin under alkaline condition. Chitin can be sourced and extracted from a diverse range of natural organisms, including molluscs, fungi, insects, crustaceans and algae [6]. Chitin exists in three different allomorphic forms depending on the sources of the compound. Most chitins, including crustaceans and insect chitin are in alpha (α) form which has a two chain antiparallel structure. However, squid pen and some diatoms have beta (β) chitin which has one chain parallel structure and in gamma (γ) chitin, the biomolecular chains are arranged randomly in which two parallel chains and one antiparallel chain form the polymeric structure [7]. Alpha-chitin and chitosan are commercially available products and are produced normally from shrimp or crab shell. Chitin/chitosan from the squid pen has a β-structure that is low packed and has weak intermolecular hydrogen bonds. These properties makes it chemically more reactive compared to the heavily packed and strong molecular structure of α-chitin/chitosan from shrimp and crab shells [8–10]. In addition, β-chitin/chitosan can incorporate water molecules in its structure and forms crystalline structure leading to higher ability to uptake and hold water more than the alpha form, which is advantageous in biomedical applications [11,12]. The source of chitosan can affect its purity, molecular weight, chain length, degree of deacetylation, density, viscosity, solubility, water retention capacity and distribution of the amino/acetamide groups. All these characteristics affect the physicochemical properties of chitosan and therefore, its application [13]. A proper biocomposite should be prepared strategically in a way to have a suitable geometry and pore size, have a

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required mechanical strength, support cell attachment *in vitro*, and have tuneable biodegradable properties [14]. In this work, chitosan was isolated from endoskeleton of New Zealand arrow squid pen (*Nototodarus sloanii*) (CHS). In addition, a commercially available crab chitosan (CHC) was used for comparison with CHS. The HA and β-TCP were prepared using waste green mussel shells. Then CHS, CHC and HA, β-TCP were processed to produce biocomposites using freezing at different temperatures (−20, −80 and −196 °C) and lyophilization processes. The produced biocomposites were evaluated to scope their potential for bone tissue engineering applications. To improve the mechanical properties of the composites in this study, tripolyphosphate (TPP) was used as an ionic cross-linker and glycerol was used as plasticizing agent.

2. Materials and methods

2.1. Materials

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) and β-TCP ($Ca_3(PO_4)_2$) powders were synthesized from waste mussel shells as previously reported [15,16]. Acetic acid, ethanol, and NaOH were obtained as analytical grade from Univar (Ajax Finechem, USA) and Sigma (St. Louis, USA), respectively. The process of squid pen chitosan (CHS) preparation and fabrication of the biocomposites are displayed in Fig. 1. Dried arrow squid pens (*Nototodarus sloanii*) were used for the preparation of β-chitin (obtained from Independent Fisheries Co., Christchurch, New Zealand). Commercial crab chitosan (CHC) purchased from Weseta International (Shanghai, China) was used as received.

2.2. Preparation of chitosan from squid pen

Isolation of chitosan from the squid pen was performed following a method developed by Chaussard and Domard [17] with minor modifications (Fig. 1). In brief, dried squid pens were grinded to particles ≤1 mm in diameter using Waring laboratory grinder (Waring Inc., USA), then deproteinization was carried out using 1 M sodium hydroxide (15 ml/g) at 60 °C and constant stirring using a rotary shaker for 24 h. Then, the leachate was removed by vacuum filtration and particles were washed extensively until neutral pH was achieved. The obtained highly moist extracted material was frozen at −80 °C and lyophilized using a freeze drier (Labconco FreeZone 12 Plus). Then the chitin powder was slowly added to a beaker containing 45% NaOH to obtain a solid/solvent ratio of 1:15 (w/v) and soaked at room temperature for 24 h [18]. The temperature of the reaction was maintained at 60 °C and the mixture was stirred for 10 h.

2.3. Degree of N-deacetylation

The degree of deacetylation (DDA) of chitosan (CHS) was calculated from data of elemental analysis (Carlo Erba Elemental Analyser EA 1108). DDA was calculated using Eq. (1) proposed by Xu et al. [19].

$$\mathrm{DDA}\,(\%) = 1 - \left[\frac{(\mathrm{C/N} - 5.14)}{1.72}\right] * 100, \quad (1)$$

where C/N is the ratio (w/w) of carbon to nitrogen in chitosan.

2.4. Preparation of the composite

Squid chitosan production and fabrication of the composites are shown in Fig. 1. Crab chitosan (CHC) solution (2%) was made by dissolving 5 g of chitosan in 250 ml of 1% acetic acid solution [20]. Due to the high hygroscopic nature of squid pen chitosan (CHS), the solution was very viscous at 1% and so the preparation of higher concentrations was technically difficult. To prepare 2% CHS solution, a 1% solution of chitosan dissolved in 1% acetic acid was subjected to microwave irradiation to remove excess water and achieving the desired concentration of 2%. The solution was then mixed by an overhead mixer (IKA T25 Ultra Turrax) for 2 min to obtain a transparent gel. The HA and β-TCP powders were mixed together based on ratios shown in Table 1. The powders were weighed and made into a homogeneous paste using ethanol (1:10, w/v). The paste was added to the chitosan solutions, homogenized by the overhead mixer. The chitosan solutions were then sonicated (Elmasonic S40 (H)) for 1 h to remove any air bubbles. The air bubble free mixtures were transferred to 15 ml Poly-Cons® plastic container and frozen at −20 °C, −80 °C, or −196 °C (the latter by direct immersion of the plastic container into liquid nitrogen for approximately 10 s). Then the samples were freeze-dried for 48 h (Labconco FreeZone 12 Plus) to form the HA/β-TCP/CH (CHC or CHS) composites. The dried HA/β-TCP/CH composites were soaked in 2.5% tripolyphosphate (TPP) aqueous solution at 4 °C for 2 h [21]. Then the composites were rinsed with deionised water for 12 h at 4 °C to remove residual TPP and were freeze dried for 24 h at −40 °C. Composites made with crab chitosan and processed at −20 and −80 °C were denoted as A220 and A280 respectively, and those made with squid pen chitosan were denoted as B220 and B280, respectively.

Table 1
Composition of HA/β-TCP/CH composites.

Composite	Compound (%)			
	HA	β-TCP	CHS	CHC
A	30	20	–	50
B	30	20	50	–

2.5. Characterization of the composites

The distribution of HA/β-TCP in the chitosan matrix was analysed using an X-Ray Diffractometer (XRD; PANnalytical X'Pert PRO MPD System) in the range $0° < 2\theta < 60°$ with Cu Kα radiation ($k = 0.15418$ nm) with a scan speed of 2.63 s [22]. The functional groups of the samples were identified using Fourier Transform Infrared Spectroscopy (FT-IR; Perkin-Elmer #100) in the region 400–4000 cm^{-1} with 4 cm^{-1} spectral resolution using the KBr pellet technique [22]. Thermogravimetric analysis of the composites was carried out using a TGA instrument (TGA; Q 500) up to 1000 °C at a 10 °C/min heating rate under a nitrogen flow. Scanning electron microscopy (SEM) (JEOL 6700F FESEM JEOL Ltd, Tokyo, Japan) was used to examine the microscopic details of the composites.

2.6. Mechanical testing

The mechanical properties of the HA/β-TCP/CH composites were tested according to the guidelines of ASTM D5024-95a (22). The mechanical properties of composites were determined using a TA.XTPlus, Texture Analyzer (Texture Technologies Corp., Stable Micro Systems, Godalming, Surrey, UK). The analysis was carried out on cylindrical samples with dimensions of 25 mm diameter × 12 mm height. A 250 N load cell was operated at a rate of 0.5 mm min^{-1} until the sample was compressed to 50% of the original height at room temperature. The compression stress–strain curves were recorded, and compression modulus, yield and ultimate strength were calculated using the Exponent software (version V6.1.5.0) using 5 replicates per each of the composite samples [23].