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Preparation and characterization of carbon nanotube-grafted-chitosan – Natural hydroxyapatite composite for bone tissue engineering

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ABSTRACT

Porous, biodegradable and biocompatible chitosan, chitosan with natural hydroxyapatite derived from *Thunnus Obesus* bone (chitosan/HAp) and chitosan grafted with functionalized multiwalled carbon nanotube in addition to HAp (*f*-MWCNT-*g*-chitosan/HAp) scaffolds were prepared for the first time via freeze-drying method and physiochemically characterized as bone graft substitutes. The cross-linkages in the novel *f*-MWCNT-*g*-chitosan/HAp scaffold were observed by FT-IR spectroscopy. The water uptake, retention ability and degradation of composite scaffolds decreased whereas thermal stability increased with an addition of HAp and *f*-MWCNT. Uniform dispersion of HAp and *f*-MWCNT in chitosan matrix with interconnected porosity of 70–200 μm (chitosan/HAp) and 46–200 μm (*f*-MWCNT-*g*-chitosan/HAp) was observed by X-ray diffraction, scanning electron microscopy and optical microscopy. Cell proliferation in composite scaffolds was twice than in pure chitosan when checked *in vitro* using MG-63 cell line. These observations suggest that the novel chitosan/HAp and *f*-MWCNT-*g*-chitosan/HAp composite scaffolds are promising biomaterials for bone tissue engineering.

1. Introduction

Over the last four decades, there is a growing interest in the field of artificial organ material preparation, transplantation, surgical reconstruction and the use of artificial prostheses to treat the loss or failure of an organ or tissue. Autograft and allograft are considered ultimate for bone grafting procedure providing osteoconductive and osteoinductive growth factors. However, limitations in donor site, additional surgery, disease transmission and expenditure (Cole et al., 2005; Giannoudis, Dinopoulos, & Tsiridis, 2005) poses a need to develop alternatives to autograft and allograft.

Chitosan, a linear polysaccharide derived from partial deacetylation of chitin, commonly found in shells of marine crustaceans, insects and cell walls of fungi, has been considerably employed in orthopedic applications due to its high biocompatibility, biodegradability, porous structure, suitability for cell ingrowth, osteoconduction and intrinsic antibacterial nature (Di Martino, Sittinger, & Risbud, 2005). The advantage of degradable polymeric implants eliminates the need for a second operation and accelerates new bone growth (Hu, Li, Wang, & Shen, 2004). Low interconnected porosity for cell attachment and mechanical strength of chitosan-based composite biomaterials needs to be improved. However, single component system cannot assist and mimic all the properties of bone and hence developing multicomponent system as an alternative for bone repair becomes mandatory.

Calcium phosphate mineral, hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] (HAp) is considered to play a vital role in various fields including spinal fusion, craniomaxillofacial reconstruction, bone defects, fracture treatment, total joint replacement (bone augmentation) and revision surgery (Best, Porter, Thian, & Huang, 2008; Palmer, Newcomb, Kaltz, Spoerke, & Stupp, 2008). Several authors reported that carbonated HAp has higher osteoconduction, bioresorption and biocompatibility (Landi, Celotti, Logroscino, & Tampieri, 2003; Orr, Villars, Mitchell, Hsu, & Spector, 2001) as compared to synthetic HAp. When compared to natural HAp, the composite of chitosan with synthetic HAp is more widely used as a bone graft substitute (Thein-Han & Misra, 2009). Natural HAp derived from pig bone combined with chitosan has been investigated by some researchers for bone regeneration (Tang et al., 2008; Yuan, Chen, Lü, & Zheng, 2008), no marine source of HAp has been explored so far.

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Recently some tricomponent systems have also been developed with improved cell proliferation on the composite scaffolds as compared to chitosan scaffold. Several authors have addressed the use of tricomponent system for bone tissue engineering including poly(methyl methacrylate) (Kim et al., 2004), gelatin (Li, Chen, Yin, Yao, & Yao, 2007), collagen (Zhang, Tang, Zhang, Xu, & Wang, 2010), poly (L-lactide acid) (Niu, Feng, Wang, Guo, & Zheng, 2009), polycaprolactone (Xiao, Liu, Huang, & Ding, 2009), carboxymethyl cellulose (Jiang, Li, & Xiong, 2009), polyamide 66 (Mu et al., 2006) and montmorillonite (Katti, Katti, & Dash, 2008) with chitosan/HAp.

Despite high mechanical properties for bone tissue growth, collagen formation and biocompatibility (Nien & Huang, 2010), to the best of our knowledge, there are no reports on chitosan grafted with CNT in additon to natural HAp for improved biocompatibility and cell growth for bone tissue engineering which may be because of cytotoxicity issues (Spear & Cameron, 2008). This study aims at incorporating carbonated natural HAp (derived from *Thunnus Obesus* bone) and *f*-MWCNT with chitosan matrix, and to use it as a potential approach to enhance the interconnected porosity and thermal stability, increase the cell proliferation and controllable degradation rate.

2. Materials and methods

Chitosan powder (degree of deacetylation 70–90%, with molecular weight 500 kDa) was purchased from Wako Pure Chemical Industries Ltd., Japan. Hydroxyapatite was isolated from *Thunnus Obesus* bone with thermal calcination method (Lee, Choi, Jeon, Byun, & Kim, 1997). Multiwalled carbon nanotube (outer diameter <8 nm, length 10–30 μm) was purchased from cheap tubes.com, USA. Human osteosarcoma (MG-63) cell line was obtained from American Type of Culture Collection (Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Gibco BRL, Life Technology (USA). MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from molecular probes (Eugene, OR, USA). Other reagents used in this experiment were all of analytical grade.

2.1. Isolation of HAp from Thunnus Obesus

The tuna bone was washed with hot water for 2 days to remove the traces of meat and skin. The washed bones were mixed with 1.0% sodium hydroxide and acetone to remove protein, lipids, oils and other organic impurities (bone and sodium hydroxide solid/liquid ratio was 1:50). After thorough washing the bones were ground in a mortar pestle and then dried at 60 °C for 24 h. In thermal calcination method, 2 g of tuna bone was placed in a silica crucible and subjected to a temperature of 900 °C in an electrical muffle furnace for 5 h (Lee et al., 1997).

2.2. Functionalization and purification of carbon nanotubes (CNTs)

Functionalization of the CNTs was carried out the procedure described by An, Nam, Tan, and Hong (2007).

2.3. Preparation of scaffold materials

2.3.1. Chitosan scaffold preparation

1.74 g of high molecular weight chitosan was dissolved in 250 ml of 2% acetic acid solution. The solution was stirred for 6 h on a mechanical stirrer (RW 20.n Labortechik) and sonicated for 1 h to remove the bubbles. This solution was then transferred to appropriate 12 well and 6 well plates with 3 g and 5 g of solution/well, respectively. The samples were further freezed at −80 °C for 5 h and lyophilized with freeze dryer to form scaffolds. The scaffolds were immersed in 10% NaOH solution for 1 day. After 1 day, scaffolds were washed with excess amount of water till the pH became neutral and lyophilized again.

2.3.2. Chitosan/hydroxyapatite (chitosan/HAp) scaffold

1.8 g of high molecular weight chitosan was dissolved in 250 ml of 2% acetic acid solution. The solution was stirred for 6 h and sonicated for 1 h to remove the bubbles. 4.2 g of naturally derived HAp was suspended in 50 ml of water and carefully transferred into the chitosan solution with the help of dropper. The solution was mechanically stirred for 48 h to disperse the HAp particle in the polymer matrix in homogeneous manner. The milky white resultant solution was then transferred to appropriate 12 well and 6 well plates with 3 g and 5 g of solution/well, respectively, and followed by the same procedure as for the chitosan scaffold.

2.3.3. Carbon nanotube-g-chitosan/hydroxyapatite (*f*-MWCNT-g-chitosan/HAp) scaffold

1.74 g of high molecular weight chitosan was dissolved in 220 ml of 2% acetic acid solution. The solution was stirred for 6 h and sonicated for 1 h to remove the bubbles. Simultaneously, *f*-MWCNT was dispersed in water and sonicated for 30 m. The dispersed *f*-MWCNT was slowly added to the stirring chitosan solution and the mixture was stirred for 6 h. At the same time, naturally derived HAp was separately dispersed in minimum amount of water. This HAp-water suspension was added slowly to the chitosan/*f*-MWCNT mixture and mechanically stirred for 48 h. Finally, this black colored mixture was transferred to appropriate 12 well and 6 well plates with 3 g and 5 g of solution/well, respectively and followed by the same procedure as for the chitosan scaffold.

2.4. General characterization

2.4.1. Porosity measurement

The total porosity was determined by the liquid displacement method (Zhang & Zhang, 2001). The procedure was as follows: first, the volume and weight of the scaffolds were measured, noted as V_0 and W_0, respectively. Secondly, the sample was immersed into the dehydrated alcohol for 48 h until it was saturated by absorbing dehydrated alcohol, and the sample was weighed again and noted as W_1.

Finally, the porosity of the sample was calculated based on the following formula

$$P = \frac{W_1 - W_0}{\rho V_0}$$

where 'ρ' represents the density of dehydrated alcohol, three parallel sets were analyzed for every scaffold and the mean value of the porosities of different scaffolds was achieved.

2.4.2. Water uptake and retention abilities

The water uptake and retention ability of scaffold was studied using the following procedure. Dry scaffolds were weighed (W_{dry}) and immersed in distilled water for 24 h. Then the scaffolds were gently removed from the beaker after 24 h and placed on a wire mesh rack. Excessive water was drained and scaffolds were weighed (W_{wet}) after 5 m to determine water uptake. To measure the water retention ability, the wet scaffolds were transferred to centrifuge tubes with filter paper at the bottom, centrifuged (Combi 514-Hanil Science Industrial) at 500 rpm for 3 m and weighed immediately (W'_{wet}). The percentage of water absorption (E_A) and water retention (E_R) of the scaffolds at equilibrium were calculated using following equation (Hu et al., 2004; Thein-Han & Misra, 2009)