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Mineralisation of chitosan scaffolds with nano-apatite formation by double diffusion technique

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Abstract

The study of inorganic crystal assembly in organic matrices has given rise to increasing interest in various fields of materials science to the natural process of biomineralisation. To mimic the formation of hydroxyapatite as natural bone, a double diffusion technique is utilised in this study to nucleate the hydroxyapatite crystals onto three-dimensional porous polymeric scaffolds. The porous polymer scaffolds were produced from chitosan by a thermally induced lyophilisation technique, which yields highly porous, well-controlled anisotropic open pore architecture. The nucleation of hydroxyapatite crystals was initiated at ambient conditions on the surface of the polymer scaffold, which was in contact with a calcium solution chamber, due to diffusion of phosphate ions through the scaffold. The morphology of the mineralised scaffold as analysed by scanning electron microscopy shows that apatite crystals were not only formed on the surface of the scaffold, but also in the pore channels and attached to the pore walls. The X-ray diffraction and Fourier transformed infrared analyses confirmed the phase purity of the formed apatite crystals. The transmission electron microscopy analysis reveals the microstructure of the entangled nano-apatite in the chitosan polymeric matrix. The in-vitro cytocompatibility tests with osteoblast-like cells (Saos-2) demonstrated that the biomineralised scaffold is a suitable substrate for cell attachment and migration in bone tissue engineering.

Keywords: Chitosan; Scaffold; Nano-hydroxyapatite; Double diffusion; Biomineralisation

1. Introduction

Bone regeneration research is needed to deal with various clinical bone diseases such as bone infections, bone tumours and bone loss by trauma [1]. Recent research advances in bone regeneration in tissue engineering have focussed on the development of three-dimensional (3D) porous scaffolds that can serve as a support, reinforce and in some cases organise the tissue regeneration or replacement in a natural way [2]. The 3D macro porous scaffolds play an important role in the formation of new tissues and provide a temporary scaffold to guide new tissue in-growth and regeneration [3–6]. Therefore the scaffold is a key component of tissue engineering [7]. The study of inorganic crystal assembly in or on an organic polymer matrix is an important focus of biomineralisation to produce nano-composites, which can mimic natural bone.

Chitosan, a natural biopolymer, is a potential candidate for tissue engineering and drug delivery systems. It is one of the most abundant naturally occurring polysaccharides, primarily obtained as a sub-product of seafood, containing amino and hydroxyl groups. The primary unit of chitin is 2-acetamido-2-deoxy-D-glucose, while that of chitosan is 2-amino-2-deoxy-D-glucose with β,1-4 glucosidic linkages [8]. Chitosan is insoluble in water, alkali and many organic

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solvents but soluble in many dilute aqueous solutions of organic acids, of which most commonly used are formic acid and acetic acid. Apart from being bioresorbable it is biocompatible, non-toxic, non-antigenic and biofunctional [9]. Chitosan has been proposed to serve as a non-protein matrix for 3D tissue growth. Chitosan could provide the biological primer for cell-tissue proliferation and reconstruction. One of the most promising features of chitosan is its excellent ability to be processed into porous structures for use in cell transplantation and tissue regeneration. In tissue engineering, the porous structure of chitosan provides a scaffold for bone cells to grow in and seed new bone regeneration. For rapid cell growth, the scaffold must have optimal micro architecture such as pore size, shape and specific surface area [10]. Therefore the major goal in fabricating scaffolds for bone tissue engineering is to accurately control pore size and porosity. Porous chitosan structures can be formed by freezing and lyophilising chitosan–acetic acid solutions in suitable moulds. Pore orientation can be directed by controlling the geometry of thermal gradients during freezing. The use of chitosan scaffolds in tissue engineering has been reported and a porous chitosan matrix has been suggested as a potential candidate for bone regeneration due to its biological and physical properties [11].

Calcium phosphates, especially hydroxyapatite (HA), having a similar chemical composition to that of the mineral phase of bone, are excellent candidates for bone repair and regeneration and have been used in bone tissue engineering for two decades. Though HA ($Ca_{10}(PO_4)_6(OH)_2$) is bioactive and osteoconductive, its mechanical properties are inadequate, making it unable to be used as a load bearing implant [12–14]. In order to achieve controlled bioactivity and biodegradability, polymer–ceramic composites have been proposed. Polymers such as chitosan have a higher degradation rate than bioceramics. Incorporation of HA into a chitosan polymer matrix has also been shown to increase osteoconductivity and biodegradability with significant enhancement of mechanical strength [15]. To combine the osteoconductivity of calcium phosphate and good biodegradability of polymers, composites have been developed for bone tissue engineering either by directly mixing the components or by a biomimetic approach [16–19]. Polymer–ceramic composite scaffolds are expected to mimic natural bone, in the way that natural bone is also a composite of inorganic compounds (calcium phosphates especially substituted carbonated hydroxyapatite) and organic compounds (collagen, protein matrix, etc.). The hydroxyapatite–chitosan–alginate porous network has been reported and demonstrated to be suitable for bone tissue engineering applications using osteoblast cells [20].

The formation of octacalcium phosphate and fluoroapatite crystals in gelatine xerogels by a biomimetic method has been reported in the literature [21,22]. The calcium phosphate–chitosan composites have been reported as being used for drug delivery systems [23–25]. The objective of the present study was to produce a 3D network of chitosan polymer in which apatite nano-crystals can nucleate and form a composite. Therefore, an attempt was made to mimic the formation of nano-sized hydroxyapatite crystals in natural bone and to fabricate a novel 3D composite consisting of porous chitosan scaffold with oriented nano-hydroxyapatite crystal growth at room temperature. The pore structure, morphology and physio-chemical characterisation were investigated. The cytocompatibility of the scaffold was investigated by determining the function and proliferation of osteoblast-like cells on it.

2. Materials and methods

2.1. Materials

Chitosan (MW 150,000, 85% deacetylation) was obtained from Fluka Chemicals. Chitosan was purified as described in the literature [20]. Briefly, the chitosan was dissolved in 2% acetic acid aqueous solution until a homogeneous 1% chitosan solution was obtained. This solution was neutralised to pH 9.0 with a 10% NaOH solution to precipitate chitosan and the precipitate was then washed well with deionised water and dried. Calcium chloride, sodium dihydrogen phosphate, acetic acid, sodium hydroxide and tris buffer used were of analytical grade from Sigma Aldrich, Germany.

2.2. Preparation of chitosan scaffold

Chitosan sponges were prepared by the freeze-drying method. Chitosan solutions with a concentration of 2 wt.% were prepared by dissolving chitosan in 1% acetic acid solution. The mixture was stirred at 50 °C for 5 h to obtain a homogeneous viscous polymer solution and filtered to remove air bubbles trapped in the viscous solution. The solution was cast in the mould and rapidly transferred into a freezer (NAPCOIL UF 400 ultra-low temperature freezer, USA) preset at a temperature of −80 °C to solidify the solvent and to induce liquid–liquid phase separation. The solidified mixture was maintained at the same temperature for 2 h. The frozen polymer was lyophilised in a freeze-dryer (Lyovac GT 2, Amsco–Finn–Aqua GmbH, Germany) at a temperature of −35 °C (0.05 mm Hg) for at least 2 days to completely remove the solvent and dried at room temperature for 12 h. The prepared sponges were then treated with 10% alcoholic sodium hydroxide solution to neutralise the acetate groups remaining on the sponge, rinsed extensively with deionised water and stored under vacuum.

2.3. Mineralisation treatment

Mineralisation of the chitosan sponges was carried out using a modified double diffusion chamber used for crystallisation as reported by Falini et al. [21] and Busch et al. [22]. The chamber contains two parts separated by a circular hole at the centre, at which the polymer scaffold can be