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Controllable synthesis and characterization of porous polyvinyl alcohol/hydroxyapatite nanocomposite scaffolds via an *in situ* colloidal technique

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ABSTRACT

During the last decades, there have been several attempts to combine bioactive materials with biocompatible and biodegradable polymers to create nanocomposite scaffolds with excellent biocompatibility, bioactivity, biodegradability and mechanical properties. In this research, the nanocomposite scaffolds with compositions based on PVA and HAp nanoparticles were successfully prepared using colloidal HAp nanoparticles combined with freeze-drying technique for tissue engineering applications. In addition, the effect of the pH value of the reactive solution and different percentages of PVA and HAp on the synthesis of PVA/HAp nanocomposites were investigated. The SEM observations revealed that the prepared scaffolds were porous with three dimensional microstructures, and *in vitro* experiments with osteoblast cells indicated an appropriate penetration of the cells into the scaffold's pores, and also the continuous increase in cell aggregation on the scaffolds with increase in the incubation time demonstrated the ability of the scaffolds to support cell growth. According to the obtained results, the nanocomposite scaffolds could be considered as highly bioactive and potential bone tissue engineering implants.

1. Introduction

In the past decade, bone tissue engineering provided new medical therapy as an alternative to conventional transplantation methods using polymeric biomaterials with or without bioactive materials such as bioceramics, bioactive glasses or glass ceramics [1,2]. Also, designing and fabricating these nanocomposite scaffolds for bone regeneration from different biodegradable polymers and bioactive materials is an essential step to engineer bone tissues. On the other hand, bone tissue engineering is a rapidly developing discipline with the intension to repair, replace or regenerate injured bone tissues. In order to repair large bone defects, porous nanocomposite scaffolds provide more advantages than the normal powder or granules, because ideal porous scaffolds consist of an interconnected macroporous network to allow cell migration, nutrient delivery, bone ingrowth and eventually vascularization [3]. A variety of materials for the scaffold preparation have been reported for bone regeneration, such as biodegradable polymers [4], HAp [5,6], bioactive glassess [7] and so on. Among bioactive materials, HAp has gained much attention because of its biocompatibility, bioactivity, osteoconductivity and osteoproductivity. Therefore, when HAp nanoparticles are implanted in human body, a new apatite layer can be formed on the surface of it, which chemically bonds with living bone [8,9].

In the context of creating more effective bioactive scaffolds, applying bioceramics is one of the most famous methods to improve scaffolds for bone repairing, and bioceramics based on calcium phosphates (CaP) particularly in the composition of tricalcium phosphate [TCP: $Ca_3(PO_4)_2$] and HAp [$Ca_{10}(PO_4)_6(OH)_2$] have extensively been studied and clinically used, and biomaterials research field focus on the synthesis of these ceramics for three decades to applications in orthopedics and dentistry [10–12]. Previous applications of porous HAp include graft applications in maxillofacial surgery as alveolar ridge augments and as bone defect filler. However, HAp is difficult to handle and keep in defect sites because of its brittleness and low plasticity. As a biocompatible, bioactive and osteoconductive material, HAp has been the first choice for bone tissue engineering scaffold compartments. Unfortunately, fabricated materials based solely on HAp cannot be used in load-bearing applications due to the low mechanical properties of HAp [13–17].

Introducing a polymeric component into HAp in order to form an organic–inorganic nanocomposite is a method most commonly used to overcome the mechanical weakness of HAp-based scaffolds. Among biodegradable and biocompatible polymers, PVA is one of the most commonly used polymers in the case of biomedical applications [18–20]. PVA has a hydroxyl pendant group on every second carbon atom on its backbone. Such a high concentration

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of hydroxyl pendant groups makes PVA uniquely capable of being cross-linked physically and without the incorporation of any chemical additives [21–23]. The physical cross-linking process of PVA (known as the freeze–thaw process) may be applied by freezing the samples at a temperature around −30 °C and subsequently thawing the samples at ambient temperature. The more freeze–thaw cycles applied to the sample, the more hydrogen bonds will be established among hydrogen and oxygen atoms in two parallel PVA polymeric chains. The formation of such bonding among polymer chains initiates crystal clusters known as "crystallites" which are randomly dispersed among an amorphous background. Such crystallite clusters have a crystallographic nature [24]. Featuring such a relative simple cross-linking mechanism which avoids the necessity to use chemical cross-linkers, PVA is an attractive candidate for biomedical applications.

In this study, we report the incorporation of PVA with HAp via an *in situ* formation of HAp crystals in a PVA solution to form biodegradable and biocompatible nanocomposite scaffolds, and a detailed study of the effect of the pH value of the reactive solution on the synthesis of nanocomposites was done because during the *in situ* synthesis of HAp nanoparticles inside the PVA solution, the pH value would affect both the formation of HAp and the incorporation of PVA by affecting the hydroxyl pendant groups. Thus, these kinds of scaffolds with well-defined macropores were first prepared by using an *in situ* colloidal technique combined with freeze-drying technique. In addition, the biocompatibility of the scaffolds were tested using human osteoblast-like cells to investigate cell contact, attachment, differentiation and subsequent adhesion of anchorage-dependent cells that soberly influence integration with tissue and eventual success or failure of a broad range of biomaterials in tissue engineering. We hypothesized that such PVA/HAp scaffold would provide not only excellent biocompatibility but also advantageous scaffolding properties, such as enhanced cell adhesion and proliferation as well as enhanced mechanical properties.

2. Materials and methods

2.1. Preparation of PVA/HAp nanocomposites

The colloidal HAp nanoparticles were synthesized via a co-precipitation method inside the PVA aqueous solution. Typically, the PVA (M_w = 72000, Merck) 7.5% (w/v) aqueous solution was prepared and then calcium nitrate tetrahydrate (Merck) were added to it. The mixture stirred for 3 h at 70 °C to obtain a PVA/calcium nitrate homogonous solution. To initiate the synthesis process of HAp nanoparticles, a separately prepared de-ammonium hydrogen phosphate 0.24 M solution (Biolab) was slowly introduced to the PVA/calcium nitrate solution through a drop-wised manner. Immediately, after addition of phosphate precursor to the solution, the synthesis process of HAp nanoparticles were commenced, and the formation of HAp nanoparticles causes the solution pH value descend, as HAp synthesis is more enhanced in basic solutions, a NaOH 1 M solution was added to the reaction vessel to control the pH value. To compare the affect of the value of pH, 3 different pH values of 7, 9 and 11 were chosen. The prepared nanocomposites underwent a single freeze–thaw cycle, including 15 h at −30 °C and 9 h thaw at ambient temperature. The prepared sample was stored in a freeze-dryer adjusted at 50 °C for 16 h.

2.2. Preparation of SBF solution

The SBF solution was prepared by dissolving reagent-grade NaCl, KCl, $NaHCO_3$, $MgCl_2{\cdot}6H_2O$, $CaCl_2$ and KH_2PO_4 into distilled water and buffered at pH 7.25 with TRIS (trishydroxymethyl aminomethane) and 1 N HCl solution at 37 °C. Its composition is given in Table 1 and is compared with the human blood plasma.

Table 1
Ion concentrations of simulated body fluid (SBF) and human blood plasma.

Ion	Plasma (mmol/l)	SBF (mmol/l)
Na^+	142.0	142.0
K^+	5.0	5.0
Mg^{+2}	1.5	1.5
Ca^{+2}	2.5	2.5
Cl^-	103.0	147.8
HCO_3^-	27	4.2
HPO_4^{-2}	1.0	1.0
SO_4^{-2}	0.5	0.5

2.3. Sample characterization

2.3.1. Transmission electron microscopy (TEM)

TEM studies were performed with the Philips CM120 operated at 100 kV. The morphology and size of the synthesized HAp nanoparticles assessed using TEM. For TEM analysis, the HAp nanoparticles were ultrasonically dispersed in ethanol for 15 min to form very dilute suspensions, and then few drops were deposited on the carbon-coated copper grids.

2.3.2. XRD analysis

The samples were analyzed by XRD with Siemens-Brucker D5000 diffractometer. This instrument works with voltage and current settings of 40 kV and 40 mA respectively and uses Cu-Kα radiation (1.540600 Å). For qualitative analysis, XRD diagrams were recorded in the interval $10^\circ \leq 2\theta \leq 50^\circ$ at scan speed of 2°/min.

2.3.3. FTIR analysis

The samples were examined by FTIR with Bomem MB 100 spectrometer. For IR analysis, 1 mg of the scraped samples were carefully mixed with 300 mg of KBr (infrared grade) and palletized under vacuum. Then, the pellets were analyzed in the range of 500–4000 cm^{-1} with 4 cm^{-1} resolution averaging 120 scans.

2.3.4. SEM analysis

The morphology and microstructure of the synthesized nanocomposite samples were evaluated using SEM analysis. The nanocomposite samples were coated with a thin layer of Gold (Au) by sputtering (EMITECH K450X, England) and then the morphology of them were observed on a scanning electron microscope (SEM-Philips XL30) that operated at the acceleration voltage of 15 kV.

2.3.5. EDX analysis

Energy dispersive X-ray analyzer (EDX, Rontec, Germany) connected to SEM was used to investigate semi-quantitatively chemical compositions.

2.3.6. Mechanical behavior

Mechanical behavior of the prepared nanocomposites was investigated by conducting compression strength test according to ASTM F 451-86. The cylindrical specimens were cut to an appropriate size (5 mm in diameter and 10 mm in thickness). The diameter and the thickness of samples were checked with an electric digital caliper. Break strength of the scaffolds were tested by Roel-Amstel with a drawing rate of 1 mm/min. Each test has been repeated five times and the average amount and standard deviation (SD) of related parameters such as E (Young Modulus) was determined.