Materials Science and Engineering C 36 (2014) 206–214

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

In vitro response of human osteoblasts to multi-step sol–gel derived bioactive glass nanoparticles for bone tissue engineering

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ARTICLE INFO

Article history:
Received 23 September 2013
Received in revised form 28 November 2013
Accepted 7 December 2013
Available online 15 December 2013

Keywords:
Tissue engineering
Nanoparticles
Bioactive glass
Sol–gel
Hydroxyapatite
Osteoblast

ABSTRACT

A multi-step sol–gel process was employed to synthesize bioactive glass (BG) nanoparticles. Transmission electron microscopy (TEM) revealed that the BG nanoparticles were spherical and ranged from 30 to 60 nm in diameter. In vitro reactivity of the BG nanoparticles was tested in phosphate buffer saline (PBS), Tris-buffer (TRIS), simulated body fluid (SBF), and Dulbecco's modified Eagle's medium (DMEM), in comparison with similar sized hydroxyapatite (HA) and silicon substituted HA (SiHA) nanoparticles. Bioactivity of the BG nanoparticles was confirmed through Fourier transform infrared spectroscopy (FTIR) analysis. It was found that bone-like apatite was formed after immersion in SBF at 7 days. Solutions containing BG nanoparticles were slightly more alkaline than HA and SiHA, suggesting that a more rapid apatite formation on BG was related to solution-mediated dissolution. Primary human osteoblast (HOB) cell model was used to evaluate biological responses to BG nanoparticles. Lactate dehydrogenase (LDH) cytotoxicity assay showed that HOB cells were not adversely affected by the BG nanoparticles throughout the 7 day test period. Interestingly, MTS assay results showed an enhancement in cell proliferation in the presence of BG when compared to HA and SiHA nanoparticles. Particularly, statistically significant ($p < 0.05$) alkaline phosphatase (ALP) activity of HOB cells was found on the culture containing BG nanoparticles, suggesting that the cell differentiation might be promoted by BG. Real-time quantitative PCR analysis (qPCR) further confirmed this finding, as a significantly higher level of RUNX2 gene expression was recorded on the cells cultured in the presence of BG nanoparticles when compared to those with HA and SiHA.

1. Introduction

The current gold standard for bone regeneration treatment is autografting, whereby the patient's own bone is harvested and implanted into the trauma site of the patient [1]. However, with the limited availability of autologous bone, and a rise in demand for orthopedic implants, the need for new bone regenerative materials has become an element of global significance. One attractive method of addressing this issue has been the use of bone tissue engineered 3D scaffolds, which act as 3D platforms for bone morphogenesis [2–4]. Conventionally, a bioceramic is used as a filler material in a polymeric matrix to improve on structural properties and induce bioactivity to encourage effective biological interaction [5]. Other methods include the use of bioceramics as bioactive coatings on inert materials, particularly for implants required in load-bearing situations, where a thin bioactive layer is deposited onto the bioinert materials.

Over the decades, there has been considerable research into bioceramics for biomedical applications, ranging from the bioinert ceramics such as zirconia and alumina to the bioactive ceramics such as bioactive glasses and calcium phosphate ceramics [6]. Bioinert materials are typically biologically inactive and incapable of forming a direct bond with the host tissue. On the other hand, bioactive materials are capable of promoting bone formation through chemical reactivity with its surroundings. The focus would therefore be on bioactive glasses or ceramics as they form bone-like apatite on the surface in vivo and in vitro, thus capable of bonding directly to tissue material [7–9]. Hydroxyapatite (HA), which has a calcium to phosphorus (Ca/P) ratio of 1.67, has been extensively used in biomedical applications due to its chemical similarities to the inorganic components of bone [10–13]. More recently, silicon substituted hydroxyapatite (SiHA) has become an attractive alternative due to its increased bioactivity and osteoconductivity [11,14,15]. Reports have suggested that, with the inclusion of trace amounts of silicon in HA, improvements to osteoblast attachment, proliferation and differentiation were observed [12]. In vivo studies carried out by Patel et al. [11] further showed that SiHA had enhanced bioactivity when compared to HA.

Bioactive glasses have also been seen as a set of promising biomaterials for various biomedical applications. The Class A properties of bioactive glasses set it apart from HA, wherein the former has both osteoinductive and osteoconductive properties whilst the latter only being osteoconductive [6,16]. Studies have shown that the high levels

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http://dx.doi.org/10.1016/j.msec.2013.12.009

of bioactivity of bioactive glasses were due to their ability to rapidly form a hydroxycarbonate apatite (HCA) layer [17]. Although important in the process of bone regeneration, it has been found that the dissolution products of Si and Ca ions from the degradation can up-regulate and activate genes which enhance osteoblastic proliferation and differentiation, leading to rapid bone regeneration [18–20]. First introduced in 1969, the classical melt-derived Bioglass® 45S5 has been widely studied and successfully used in numerous commercial products [4,16]. However, the melt-derived process, which involves the quenching of oxides at high temperature to obtain this glass, has its limitations such as requiring high processing temperature and a narrow Class A compositional range. To overcome this, bioactive glasses were synthesized by the sol–gel process [21]. The sol–gel process is a relatively low temperature method of obtaining bioactive glasses from the hydrolysis and polycondensation of metal hydroxides, alkoxides and inorganic salts. Further benefits such as better bone bonding ability, higher degradation rates and the ability to incorporate various cation inclusions into the sol–gel network have made this method of bioactive glass synthesis very attractive [22,23].

Studies have shown that fundamental changes in pH, precursor concentration and processing temperature have been able to change the silica networks and hence affect the final glass structure [24,25]. During the sol–gel reaction, an acidic reaction leads to the formation of a linear or random branched polymer, whilst under a basic reaction, clustered polymer branches are formed [25,26]. Stöber et al. [24] demonstrated the use of basified water as a morphological catalyst to successfully synthesize monodispersed silica particles from the acidic hydrolysis of tetraalkyl silicates. Expanding on the usage of a basified catalyst to obtain homogenous sized and shaped particles, Hong et al. [27,28] successfully synthesized bioactive glass nanoparticles in the range of 30–100 nm diameters through a sol–gel and co-precipitation method.

Nanomaterials which are one dimension less than 100 nm are classified as nanoparticles [29]. As a comparison to living organisms, proteins are typically 5 nm in size whilst organelles fall in the 100–200 nm domain [30]. The potential benefits of nanoparticles are their inherent high surface to volume (S/V) ratios, allowing increased solubility, and hence increased bioactivity. Recent research on the mesoporosity of BG nanoparticles also point towards their potential as platforms for drug delivery and imaging [31]. Employing sol–gel synthesis of bioactive glass with an alkali morphological catalyst, this study firstly sets out to obtain BG nanoparticles of a homogenous shape and size, allowing for a narrow size distribution. Subsequently, the in vitro response and bioactivity of these biomaterials were tested with physiological fluids of phosphate buffer saline (PBS), Tris-buffer (TRIS), simulated body fluid (SBF) and Dulbecco's modified Eagle medium (DMEM). It was crucial to understand the cellular responses of primary human osteoblast (HOB) cells in culture with BG nanoparticles, such as cytotoxicity, cell proliferation and cell differentiation, which were investigated in this study for their potential application in coatings and scaffolds for bone tissue engineering.

2. Materials and methods

2.1. Material preparation

BG nanoparticles of composition 58% SiO_2 – 37% CaO – 5% P_2O_5 (mol%) were synthesized by modifying the method described by Hong et al. [28]. Briefly, 21.38 g of tetraethyl orthosilicate (TEOS; Aldrich) was dissolved in 120 mL of ethanol with the pH adjusted to pH 1.9 using 0.1 M nitric acid. Separately, 15.46 g of calcium nitrate ($Ca(NO_3)_2$; Aldrich) was dissolved in 200 mL of deionized water and then mixed together with the TEOS solution. 1.164 g of ammonium dibasic phosphate ($(NH_4)_2HPO_4$; Aldrich) was dissolved in 3 L of deionized water, the pH was adjusted to pH 11 using ammonium hydroxide (NH_3 28% in H_2O; Aldrich). Using a peristaltic pump, the solution containing TEOS and calcium nitrate was slowly dripped into the ammonium dibasic phosphate solution and stirred vigorously. During this process, the pH value of the solution was maintained at pH 11 using ammonium hydroxide. The precipitate obtained was aged for 48 h and dried in the oven at 90 °C. The precipitate was then crushed and sintered at a temperature of 680 °C to eliminate any residual substances of nitrates and silanol groups [32].

HA and SiHA nanoparticles were synthesized using the precipitation method in which 1.953 g of calcium hydroxide ($Ca(OH)_2$; Aldrich) and 1.717 g of phosphoric acid (H_3PO_4 85% concentrate; Aldrich) was dissolved/diluted in 200 mL distilled water respectively. For SiHA containing 1.0 wt.% Si, 0.197 g TEOS was added to the phosphoric acid and mixed homogenously. Using a peristaltic pump, the acid was slowly dripped into the calcium hydroxide solution under stirring. Ammonium hydroxide was added during the reaction to maintain the pH above 10 [33].

2.2. Material characterization

2.2.1. Evaluation of nanoparticles

The morphology of the synthesized nanoparticles was examined using a transmission electron microscope (JEOL 1010 TEM) with an accelerating voltage of 80 kV. The nanoparticles were suspended in ethanol using a sonicator (Branson 250 Sonicator) before being collected on TEM copper grids for imaging. The surface morphology and chemical composition of the nanoparticles were analyzed by a scanning electron microscope (JEOL JSM-6301F field emission SEM) equipped with an energy-dispersive X-ray (EDX) spectroscope (INCA X-sight Oxford Instruments) detector. Further quantitative chemical composition analysis was determined by X-ray fluorescence (XRF) (London & Scandinavian Metallurgical Co.). Crystal structure of the nanoparticles was analyzed by X-ray diffraction (XRD) using an X-ray diffractometer (Bruker D4 Endeavor) with copper Kα radiation using 2θ values between 5° and 80° with a 0.05° step size and a count rate of 2 s/step. A Fourier transform infrared spectroscope (Perkin Elmer 2000 FTIR) was utilized to characterize the functional groups of the biomaterials produced. The nanoparticles were crushed with potassium bromide (KBr) and then compacted into thin disk for FTIR analysis. An average of 20 scans was recorded for each spectrum, which was normalized against pure KBr.

2.2.2. In vitro testing

The nanoparticles were immersed in phosphate buffer saline (PBS), Tris-buffer (TRIS), simulated body fluid (SBF) and Dulbecco's modified Eagle medium (DMEM). PBS was prepared by dissolving one tablet (PBS; Aldrich) in 200 mL of deionized water to obtain a final pH of 7.4. TRIS buffer was made by dissolving tris(hydroxymethyl)aminomethane (Aldrich) with deionized water and adjusted to pH 7.4 using hydrochloric acid (1 M HCL; Aldrich). SBF, closely resembles the ion concentration of blood plasma, was prepared accordingly to the method reported previously [8], and adjusted to a final pH of 7.4. The nanoparticles with a final concentration of 0.1 mg/mL were incubated at 37 ºC for 1, 4, 7, 14, 21 and 28 days. At each time point, the pH of the supernatant was measured. The reacted nanoparticles in SBF were removed, rinsed in de-ionized water, and dried in an air circulation drying oven, and the changes in surface structure was analyzed by FTIR.

2.3. In vitro biological study

2.3.1. Cell culture

HOB cells, obtained by a method previously described [34] were cultured in 25 cm^2 sterile tissue culture flasks at 37 °C in a humidified air atmosphere of 5% CO_2. The culture medium used was Dulbecco's modified Eagle medium (DMEM) media, supplemented with 10% fetal calf serum (FCS), L-ascorbic acid (150 g/mL), and L-glutamine, penicillin and streptomycin (100 units/mL). Once confluent, the HOB cells were collected by trypsinizing adherent cells and resuspended in DMEM and cell viability was assessed using the Trypan blue exclusion test.