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In vitro evaluation of osteoblast adhesion, proliferation and differentiation on chitosan-TiO_2 nanotubes scaffolds with Ca^{2+} ions

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ABSTRACT

Hydrothermally synthesized TiO_2 nanotubes (TNTs) were first used as a filler for chitosan scaffold for reinforcement purpose. Chitosan-TNTs (CTNTs) scaffolds prepared via direct blending and freeze drying retained cylindrical structure and showed enhanced compressive modulus and reduced degradation rate compared to chitosan membrane which experienced severe shrinkage after rehydration with ethanol. Macroporous interconnectivity with pore size of 70–230 μm and porosity of 88% were found in CTNTs scaffolds. Subsequently, the functionalization of CTNTs scaffolds with $CaCl_2$ solutions (0.5 mM–40.5 mM) was conducted at physiological pH. The adsorption isotherm of Ca^{2+} ions onto CTNTs scaffolds fitted well with Freundlich isotherm. CTNTs scaffolds with Ca^{2+} ions showed high biocompatibility by promoting adhesion, proliferation and early differentiation of MG63 in a non-dose dependent manner. CTNTs scaffolds with Ca^{2+} ions can be an alternative for bone regeneration.

1. Introduction

Bone tissue engineering seems to be a promising technology to overcome the limitations of current bone grafts associated with limited source, immunological rejection and infection [29]. It is a technology that combines the principles of medical science and engineering to repair or recover damaged tissue by the substitution with scaffolds. Scaffold serves as an artificial extracellular matrix (ECM) which mimics the extracellular environment by providing appropriate environmental conditions for intercellular contact and signaling. With high porosity and interconnected network, scaffolds allow cell penetration and adhesion and support structure [19]. They are always embedded with growth factors and have an appropriate degradation rate which matches with bone formation [10].

With advanced technology, common materials such as synthetic polymeric materials, naturally derived polymers and composite materials have been under extensive investigation as artificial scaffolds [10]. Chitosan is a semi-synthetic polymer derived from chitin which is widely distributed in exoskeleton of insects, fungi and crustaceans. It is a favorable material for tissue engineering, as it causes minimal foreign body reaction and would not trigger inflammation and of high biodegradability [10]. However, it has to combine with other materials to form an ideal scaffold due to its low mechanical strength and osteoconductivity [28].

Metallic titanium (Ti) is frequently used for orthopedic implantation because of its low toxicity, high mechanical strength, and good biocompatibility. Ti usually has a layer of titanium dioxide (TiO_2). As Ti is bioinert, the presence of TiO_2 layer confers the necessary bone-bonding ability [5]. Especially, TiO_2 nanotubes (TNTs) washed with calcium acetate promoted a profound bone-forming ability [9,11]. It was hypothesized that TNTs with adsorbed calcium (Ca) ions probably mediate cell adhesion and promote bone formation and hence this current study was conducted. Several previous studies also proved that Ca and phosphorous (P) ions involve in osteoblast differentiation [15,18]. In fact, the concentration of extracellular Ca^{2+} ions plays an important role in the regulation of osteoblast proliferation and differentiation through calcium/calmodulin signaling pathway [30].

In the present study, the incorporation of TNTs into chitosan matrix via direct blending was attempted and expected to improve the compressive modulus of chitosan scaffolds. The biocompatibility of such newly developed scaffolds was subsequently elevated via the liquid-solid adsorption of Ca precursor solution, as TNTs with higher specific surface area were hypothesized to provide adsorption sites for Ca^{2+} ions. The adsorption affinity of new chitosan composite scaffolds towards Ca^{2+} ions was determined and verified which would facilitate

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the explanation on the profound bone-forming ability of TNTs as prepared by Kasuga [9] and Kubota et al. [11]. With the adsorbed Ca^{2+} ions, these newly developed composite scaffolds were expected to exhibit more biologically inspired properties by promoting better osteoblastic functions on osteosarcoma cell lines (MG63). In particular, the dosage effect of Ca^{2+} ions on scaffolds in terms of adhesion, proliferation and differentiation was investigated.

2. Materials and methods

2.1. Materials

Titanium dioxide (TiO_2) powder, NaOH pellets, $CaCl_2$ powder, chitosan powder (middle viscous, 80% deacetylated), sodium bicarbonate, sodium pyruvate, β-glycerophosphate, *p*-nitrophenyl phosphate (pNPP), fluorescein diacetate (FDA), propidium iodide and dimethyl sulphoxide (DMSO) were purchased from Sigma Aldrich (Germany). The solvent used was analytical grade of acetic acid, hydrochloric acid and ethanol purchased from Merck (Germany), Sigma Aldrich (Germany) and R&M chemicals (UK), respectively. Ca/Mg hardness kit was purchased from HACH (USA). Phosphate buffered saline was prepared by dissolving sodium chloride (NaCl), potassium chloride (KCl), sodium phosphate dibasic, monopotassium and phosphate which were purchased from Sigma Aldrich (Germany) in distilled water. 24-well plates and 96-well plates were purchased from Orange Scientific (Belgium). Minimum essential media (MEM), Fetal bovine serum (FBS), penicillin/streptomycin, 0.25% trypsin and 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolim bromide (MTT) were purchased from Gibco (USA). Ascorbic acid was purchased from Merck (Germany), while Pro-Prep Protein Extraction Solution was purchased from iNtRON Biotechnology (South Korea). The deionised water was filtered by Mili-Q integral water purification unit. All chemicals were of analytical grade and used without further purification.

2.2. Fabrication of chitosan and chitosan-TNTs (CTNTs) scaffolds

TNTs were synthesized via hydrothermal synthesis. Two grams of TiO_2 was mixed with 40 ml of 10 M NaOH homogenously on a hot plate (Heidolph, Germany) for 5 h before being subjected to hydrothermal reaction at 130 °C for 72 h [3]. The resultant powder was filtered and washed with deionised water and 0.01 M HCl until neutral pH. Powder was then dried at 55 °C and heated to 400 °C at 10 °C/min for 5 h. One gram of chitosan flakes was stirred with 100 ml of 0.2 M acetic acid for 3 h. One mililitre of pure chitosan solution was transferred into each well of 24-well plate and frozen at −20 °C for 24 h. For the chitosan-TNTs (CTNTs) scaffold preparation, 16 weight percent (wt%) of TNTs was directly blended with chitosan solution for 5 h. One mililitre of the colloidal mixture was also pipetted into each well of 24-well plate and stored at −20 °C for 24 h. Both solidified chitosan solution and the colloidal mixtures of chitosan and TNTs were then subjected to 24-h freeze drying at −40 °C. Scaffolds were rehydrated in a series of ethanol by immersing them with 100% ethanol, 70% ethanol and 50% ethanol for 1 h, 30 min and 30 min, respectively. The use of ethanol in the rehydration process served a purpose of sterilization which is required for cell-based evaluations. Scaffolds were dried prior to characterization and functionalization with $CaCl_2$ solution.

2.3. Microstructure characterization of TNTs, chitosan and chitosan-TNTs (CTNTs) scaffolds

The particle size and internal structure of heated powder were verified by using Transmission Electron Microscope (Model No: Philips HMG 400 TEM, Netherlands) at an accelerating voltage of 100 kV. Surface morphology and pore size of chitosan scaffolds and chitosan-TNTs (CTNTs) scaffolds were studied by using Field Emission Scanning Electron Microscope (FESEM) at an accelerating voltage of 10-20 kV. The estimation of pore diameter was obtained by analysis of digital FESEM images. Diameter and thickness of scaffolds were measured with a Digimatic micrometer to calculate the bulk volume of scaffolds. The skeletal volume of the scaffolds was determined by adopting the measurement protocol as developed by Tan et al. [25] and measured by using a pycnometer (Laborglas, Germany). Subsequently, the porosity of each scaffold was determined using Eq. (1):

$$\text{Porosity} = \left[1 - \left(\frac{V_{\text{skeletal}}}{V_{\text{bulk}}}\right)\right] \times 100\% \quad (1)$$

where V_{skeletal} was the skeletal volume of scaffolds (cm^3) and V_{bulk} was the bulk volume of scaffolds (cm^3). Compressive modulus of scaffolds was determined using a mechanical tester (Model No. H25KS, USA). Scaffold was compressed by a load cell of 500 N applied at crosshead speed of 1 mm/min until 60% of its original thickness was achieved [31]. The compressive modulus for scaffold was obtained as the slope of initial linear part of the stress against strain plot. Scaffolds were immersed in Phosphate Buffered Saline (PBS) solution for 14 days at pH 7.2 and 37 °C [26]. The initial weight of scaffold was measured and recorded. After 14-day immersion, wet scaffold was taken out and rinsed with deionised water for three times. The rinsed scaffold was then subjected to 24-h freeze drying at -40 °C followed by weight measurement. Any weight change in the scaffold was represented as the relative weight loss. Each test was performed in triplicate and the data reported were represented as means ± standard deviations.

2.4. Functionalization of scaffolds with $CaCl_2$ solution via liquid-solid adsorption

Scaffolds were immersed in 20 ml of different concentrations of calcium chloride ($CaCl_2$) solution: 0.5 mM, 1.5 mM, 4.5 mM, 13.5 mM and 40.5 mM for functionalization. The pH of $CaCl_2$ solutions was adjusted to 7.2. Scaffolds were equilibrated in $CaCl_2$ solutions at 100 rpm for 24 h. The adsorption of Ca^{2+} ions onto scaffolds was determined by measuring the changes in concentration of solution before and after adsorption using Ca/Mg hardness kit. By using Eq. (2), the amount of Ca^{2+} ions adsorbed onto scaffolds was calculated. After functionalization, scaffolds were washed with deionised water and dried prior to in vitro tests.

$$q_e = \frac{(C_o - C_e) \times V}{M} \quad (2)$$

where q_e is the amount of Ca^{2+} ions adsorbed by per gram scaffold (mg/g), C_o and C_e are the concentrations of $CaCl_2$ solutions before and after functionalization (mg/L), respectively, V is the volume of $CaCl_2$ solution (L) and M is the dry mass of scaffold (g).

The adsorption of Ca^{2+} ions onto scaffolds were analysed by using Langmuir, Freundlich and Temkin isotherm models. Langmuir isotherm assumes monolayer adsorption on homogenous surface with finite number of adsorption sites. The mathematical representation of Langmuir isotherm is shown by Eq. (3):

$$\frac{C_e}{q_e} = \frac{1}{bq_m} + \frac{C_e}{q_m} \quad (3)$$

where q_e represents equilibrium adsorption capacity per unit weight of scaffold (mg/g), q_m is the maximum adsorption capacity of per unit weight of scaffold for Ca^{2+} ions (mg/g), C_e is the equilibrium liquid-phase concentration (mg/L) and b is the Langmuir isotherm constant (L/mg).

Freundlich isotherm is used to represent multilayer adsorption on heterogeneous surface with the frequency of site associated with free energy of adsorption decreases exponentially with the increase of the