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In vitro evaluation of osteoblastic cells on bacterial cellulose modified with multi-walled carbon nanotubes as scaffold for bone regeneration

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ABSTRACT

In this paper we explore the use of native bacterial cellulose (BC) in combination with functionalized multi-walled carbon nanotubes (MWNTs) as an original biomaterial, suitable three-dimensional (3D) scaffold for osteoblastic cell culture. Functionalized MWNTs were mixed with native BC (secreted by *Gluconacetobacter xylinus*) with the aim of reinforcing the mechanical properties of BC. The results indicate that BC-MWNTs scaffolds support osteoblast viability, adhesion and proliferation at higher levels as compared to traditional culture substrates. Chemically functionalized MWNTs are also an excellent material to be used as scaffold because these did not affect cell viability and showed an enhanced osteoblast adhesion. These results suggest the potential for this combination of biomaterials, i.e. BC and carbon nanomaterials, as scaffolds for bone regeneration.

1. Introduction

The field of tissue engineering is a multidisciplinary field that draws on experts from clinical medicine, mechanical engineering, materials science, genetics, and interrelated disciplines from both engineering and the life sciences [1]. Tissue engineering relies extensively on the use of scaffolds to provide the appropriate environment for the regeneration of tissues such as bone [2]. These scaffolds act essentially as templates for tissue formation and are typically seeded with cells and, occasionally, with growth factors, or are subjected to biophysical stimuli in bioreactors, devices or systems that apply different types of mechanical or chemical stimuli to cells [3–5]. These cell-seeded scaffolds are usually produced from a variety of biomaterials and a number of key considerations (biodegradation, biocompatibility, mechanical properties of the scaffold architecture, manufacturing technologies, etc.) must be taken into account for designing or determining the suitability of a scaffold for use in tissue engineering [6].

In recent years, natural polymers have gathered widespread interest for use in biomedical materials and devices for the various benefits they offer over synthetic polymer materials. As materials naturally present in living organisms, with properties tailored to meet specific needs of the organisms in which they reside, they often carry with them certain native properties of those respective tissues. Such natural polymers as collagen, elastin, alginate, chitosan, starch, and cellulose are widely present in natural organisms and have all been investigated for various uses in tissue engineering [7]. Among these, bacterial cellulose (BC) has gained particular interest more recently. Cellulose's virtual ubiquity in nature in organisms ranging from redwoods to plankton attests to its biological utility, and its usefulness in biomedical and tissue engineering applications is now becoming apparent as well [8,9].

BC is a polysaccharide used in the fabrication of reinforced paper [10] and in the last few years it has been investigated as a material for medical applications [11–13]. Studies carried out in vitro and in vivo have demonstrated its biocompatibility [14–16]. Due to its water absorption capacity, porosity and stability, BC alone has been used in tissue engineering of cartilage and bovine chondrocytes [17], replacement of blood vessels in rats [18], and in the wound healing process [19]. On the other hand, multi-walled carbon nanotubes (MWNTs) are also a material that has great potential for being used in tissue engineering because of its electrical and mechanical properties [20–23], but mainly because it is a material that can be easily functionalized with different chemical groups [24,25], which can help to decrease or even inhibit their inherent toxic effects [26–28].

However, each of these individual materials has specific disadvantages. In the case of BC, its mechanical properties are not optimal for bone regeneration, and producing scaffolds with adequate mechanical properties is one of the great challenges in attempting to regenerate

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bone or cartilage [29]. For these tissues, the implanted scaffold must have sufficient mechanical integrity to function from the time of implantation to the completion of the remodeling process [30]. The mechanical properties of a hydrogel are important for cell growth in the scaffolds [31]. The stiffness of hydrogels has been reported to direct the differentiation of different cell types. Because of these facts, the idea of combining BC with a material with high mechanical properties, such as MWNTs [32–34], to enhance biological and/or mechanical properties of scaffolds and at the same time removing the disadvantages of both materials is highly desirable.

The response of osteoblastic cells on the development of scaffolds made by the combination of native BC and MWNTs has been evaluated in this paper. The structure, mechanical properties and hydrophobicity of the scaffolds was characterized as well as, and the viability, proliferation, adhesion and morphology of osteoblasts was also investigated.

2. Materials and methods

2.1. Production and purification of native BC

A suspension of *Gluconacetobacter xylinus* (ATCC® 700178, LGC Standards AB, Sweden) was inoculated in 100 ml sterile petri dishes filled with 50 ml of sterile culture medium (described by Matsuoka et al. (1996)) [35]. The petri dishes were cultured for 7 days in an incubator at 30 °C under static conditions, until BC pellicles (Ø14, thickness 8 ± 1 mm) were formed. After harvest, the BC pellicles were rinsed with tap water to remove excess medium and placed in 1000 ml flasks filled with 0.5 M NaOH in a shaking bath at 37 °C under shaking motion (100 shakes per minute) for 7 days. The alkaline solution was replaced every day twice. Using the same purification conditions, the alkaline solution was replaced with deionized (DI) water until pH was neutral. In the last purification step, the BC pellicles were steam sterilized (100 kPa, 121 °C, 20 min) and stored at 4 °C until use.

2.2. Purification and functionalization of MWNTs

Crude MWNTs were purchased from Thomas Swan Advanced Materials & Co. Ltd. (Durham, UK). MWNTs were subjected to a purification process, which consisted in heating MWNTs in air at 400 °C for 30 min and then soaking them in hydrochloric acid for 12 h. Next, MWNTs were rinsed twice in DI water and once with ethanol to eliminate trapped acid and traces of amorphous carbon. The resulting solution was filtered using a 5 μm polytetrafluoroethylene membrane filter [36]. After the purification process, MWNTs were functionalized by the following oxidation procedure described [37]. 60 mg of purified MWNTs were suspended in a concentrated sulfuric and nitric acid mixture (3:1 v/v, 50 ml), and subsequently sonicated for 24 h in ultrasonic bath (200 W) at 50 °C. The resultant mixture was centrifuged for 60 min, and the resulting black sediment was washed thoroughly with deionized water; this process was repeated four times. Finally, functionalized MWNTs were rinsed and filtered in DI water with acetone and dried under vacuum for 24 h at 100 °C. Functionalized MWNTs are called MWNTs-COOH.

2.3. Scaffold fabrication

As a first step in the elaboration of scaffolds, pellicles of native BC were disintegrated by high intensity mixing until a fibrous pulp of BC was obtained. To obtain MWNTs-COOH suspensions at different concentrations, MWNTs-COOH powder was suspended in DI water at 0.5 mg/ml and 0.25 mg/ml and sonicated for 1 h at 60 °C using an Cole-Parmer 470 50 W sonicator at 45 kHz of frequency. Subsequently, homogeneous dispersions of native BC (pulp) and MWNTs-COOH (two concentrations: 0.5 mg/ml and 0.25 mg/ml) were accomplished by using a high intensity mixer with an ultrasonic tip (Ultra-Turrax) at 8000 rpm for 10 min. The amounts used for combinations of native BC and MWNTs-COOH were; 1 g of native BC pulp (1 wt%) and 100 μl of each MWNTs-COOH suspensions (0.5 mg/ml and 0.25 mg/ml). One sample of native BC pulp (1 g) with 100 μl of DI water was prepared as control. A solution of sodium alginate with D-mannitol was prepared. Alginate was dissolved to a concentration of 2.5% (w/v) in 4.6% (w/v) D-mannitol aqueous solution. D-mannitol was used to retain osmolarity. All formulations of native BC and BC-MWNTs with alginate/D-mannitol solution were prepared with proportion: 90:10. The final mixture was deposited into culture boxes. Next, $CaCl_2$ solution (90 mM) was added to each well in a concentration of 5:2 v:v. Aqueous solution of $CaCl_2$ acted as the cross-linking solution. All samples within the culture dishes were allowed to stand for 30 min before being lyophilized for 24 h to obtain cylindrical 3D structures. To obtain films or 2D-scaffolds, samples were dried at room temperature for 24 h.

2.4. Physicochemical characterization

2.4.1. MWNTs-COOH characterization

Before and after functionalization process, morphological characterization of MWNTs was performed by field emission scanning electron microscopy (FE-SEM) Inspect F50 (FEI Company). Both, crude and functionalized MWNTs were analyzed directly from the powder placed on the carbon tape.

Zeta potential (ζ-potential) was quantified with Delsa™Nano C Particle Size and Zeta Potential Analyzer (Beckman Coulter Inc.) in order to quantify the changes on the electric charge due to functional groups along the entire length of MWNTs. Functionalized MWNTs were suspended in DI water (1.0 mg/ml) and sonicated for 1 h at 60 0 °C using a Cole Parmer 470 50 W sonicator at 45 kHz of frequency. The ζ-potential of the samples was measured.

2.4.2. Scaffold surface topography analysis

SEM (FE-SEM Inspect F50 FEI Company) was used for studying the scaffold surface topography. The samples were covered with gold (the thickness of a layer was 15 nm) in a sputter coater with glow discharge.

2.4.3. Wettability properties

Wettability properties were determined by water contact angle (WCA) by the sessile drop technique using a DGD-DX goniometer; water was deposited on each scaffold surface with a micro-syringe. Images were capturing immediately after the deposition using a micro-video system (GBX). The acquired images were analyzed by Visiodrop software (GBX). The WCA values were analyzed by the Wilcoxon test.

2.4.4. Mechanical properties

Mechanical properties were investigated by means of rheology. The rheological behavior of the scaffolds was characterized using an AR2000 controlled strain rheometer from Rheometric Scientific (now TA Instruments). Each 3D-scaffold was placed on a stainless steel parallel plate array (40 mm diameter) at 0.5% strain, a frequency of 0.05 Hz, and 1 mm gap at 25 °C. The storage and loss moduli (G′ and G′) were recorded as a function of time. Only storage modulus (G′) is showed.

2.5. In vitro biocompatibility assay

2.5.1. Osteoblast culture

Osteoblasts from human inferior maxillary bone (mandible or jawbone) were cultivated. Cells were maintained in DMEM low Glucose medium (Biowest the serum specialist) supplemented with 10% fetal bovine serum (Biowest the serum specialist) and 1% antibiotic (10,000 units Penicillin, 10 mg of streptomycin and 25 μg per ml Amphotericine B by Sigma) at 37 °C in an atmosphere of 95% humidity and 5% CO_2 until use.