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Chitosan scaffolds formation by a supercritical freeze extraction process

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ABSTRACT

A crucial step of Tissue Engineering (TE) approach is the fabrication of 3-D biodegradable scaffolds. It has been achieved using various techniques, such as gas foaming, fiber bonding, solvent casting/particulate leaching, phase separation and 3D-printing. Each technique presents specific advantages and disadvantages; but, all of them share the difficulty to obtain simultaneously the macro, micro and nanostructure. In this work, a Supercritical Freeze Extraction Process (SFEP) is proposed for the formation of chitosan structures suitable for TE applications. We showed that it is possible to produce chitosan scaffolds characterized by a micrometric cellular structure, nanofibrous sub-structure and porous surfaces. The low process temperature allows to obtain 3-D solids, whose structure is preserved during supercritical drying. Preliminary results on cell cultivation confirmed that the generated chitosan scaffolds are characterized by a morphology that is potentially suitable for TE applications. A good cell adhesion was obtained and a large percentage of living cells was observed. This result can depend on the micrometric morphology of the scaffolds, that assures a good nutrient diffusion, and on the nanometric sub-structure that allows an adequate cells adhesion.

1. Introduction

Although significant advances have been made in medical techniques to repair damaged organs or tissues as a result of a trauma, accident or cancer, transplantation of organs or tissues is still a widely accepted therapy to treat patients. Autologous transplantation is limited because of donor site infection or pain to patients because of secondary surgery. Alternative tissue sources that originates from other humans (or animals) remain problematic, mainly due to immunogenic responses upon implantation and a shortage of donor organs.

Tissue engineering (TE), applying methods derived from engineering and life sciences applied to the production of artificial constructs, to direct tissue regeneration, has attracted many scientists with the hope to treat patients in a minimally invasive and less painful way [1]. Tissue engineering general aim is to isolate specific cells from a patient, to grow them in a three-dimensional (3-D) biodegradable scaffold, under controlled culture conditions, to transplant the construct to the desired site in the patient's body, and to direct new tissue formation into a scaffold that degrades over time. The first and crucial step of the TE approach is the fabrication of a 3-D biodegradable scaffold. To achieve this goal, the biodegradable matrix should possess sufficient mechanical strength, adequate 3-D structure and suitable degradation rate. Attempts at producing different biological materials (bones, cartilages, nerves, tendons, vessels, cardiac valves, skin, etc.) have been made and each tissue requires different morphological characteristics; however, some of these characteristics are common to all tissues [2]: (a) the 3-D structure has to be similar to the tissue to be substituted; (b) the material has to be characterized by a very high porosity with an open-pore geometry and suitable pore size; (c) the obtained scaffold has to be nano-structured; (d) adequate mechanical properties are necessary to maintain the predesigned tissue structure and to support the specific loadings applied to the original tissue; (e) the biocompatibility and the proper degradation rate have to be assured; (f) the inflammatory response has to be absent or reduced.

Different polymeric materials have been proposed for TE applications; for example, attention has been given to chitosan-based materials and their applications [3]. Chitosan is a linear polysaccharide, composed of glucosamine and N-acetyl glucosamine (GAG); the glucosamine/N-acetyl glucosamine ratio being referred as the degree of deacetylation. Depending on the source and preparation procedure, its molecular weight may range from 300 to over

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1000 kDa with a degree of deacetylation from 30% to 95%. In its crystalline form, chitosan is normally insoluble in aqueous solutions above pH 7; however, in dilute acids (pH 6.0), the protonated free amino groups on glucosamine, improve the solubility of the polymer [4,5]. Chitosan is biocompatible, biodegradable and allows osteoconduction [6]. Another interesting property is its intrinsic antibacterial activity. For example, some studies showed that chitosan can reduce the infection rate of experimentally induced osteomyelitis by *Staphylococcus aureus* in rabbits [7].

In the overall, chitosan offers broad possibilities for cell-based tissue engineering [8] and it has been proposed for various tissue engineering applications; for example, as a scaffolding material in articular cartilage engineering [9], due to its structural similarity with various GAGs found in articular cartilage. Iwasaki et al. [10] reported alginate-chitosan hybrid polymer fibers which showed increased cell attachment and proliferation in vitro, compared to alginate alone; these fibers also showed as increased tensile strength. Chitosan has also been proposed in bone tissue engineering, since it can promote growth and mineral rich matrix deposition by osteoblasts in culture [11]. Several studies have been focused on the use of chitosan–calcium phosphate (CP) [12] and chitosan-hydroxyapatite (HA) [13] composites for this purpose.

The fabrication of biodegradable scaffolds has been achieved using various techniques, such as gas foaming, fiber bonding, solvent casting/particulate leaching, phase separation and 3D-printing [14–16]. Each technique presents specific advantages and disadvantages; but, all of them share the difficulty to obtain simultaneously the macro, micro and nanostructure.

One of the most used methods in TE is phase separation of a polymeric homogeneous solution in a polymer-poor and a polymer-rich liquid phase [17–20]. The polymer-poor phase will grow and coalesce, forming pores in the scaffold. In Thermally Induced Phase Separation (TIPS), the lowering of the solution temperature with respect to the room temperature is used to induce the phase separation. In this process, generally a cellular porous structure is formed. If the temperature is low enough, the solid–liquid demixing mechanism can induce the separation of the frozen solvent and the concentrated polymer phase [21]. The subsequent step is the solvent removal, in which the porous structure needs to be carefully preserved: generally, freeze-drying is attempted, but, this method presents several disadvantages being time consuming and having the problem of the collapse of the structure [19,20,22,23]. However, this step is mandatory because commonly TIPS is a reversible process.

Supercritical fluids assisted processes have been proposed to overcome the limitation of traditional techniques in several fields [24–26]. Various techniques based on the use of supercritical carbon dioxide (SC-CO_2) have also been proposed in TE [27]: among them, supercritical induced phase separation (SC-IPS) [28,29], supercritical foaming [30], supercritical gel drying combined with particulate leaching [31–36], electrospinning in SC-CO_2 [37] are the most studied. Using SC-CO_2 assisted techniques, the traditional TE processes can be improved, due to the tunability of the mass transfer typical of dense gases. Moreover, the use of organic solvents is reduced and residual solvents in the products are near to zero, due to the large affinity of SC-CO_2 with almost all the organic solvents [38].

The aim of this work is to propose a *supercritical freeze extraction process (SFEP)* for the formation of porous structures suitable for TE applications. It combines the TIPS process with supercritical drying and can produce a complete and fast solvent elimination, avoiding the structure collapse. We will explore the possibility of producing 3-D chitosan scaffolds characterized by a homogeneous microstructure, suitable for cells colonization and growth, and by a nanoporous sub-structure for cells interaction and guidance for adhesion, migration and differentiation. Preliminary cells cultivation tests on human Mesenchimal Steam Cells (hMSC) will be presented to evaluate first cell interactions with the produced structures.

2. Materials and methods

2.1. Materials

Chitosan medium molecular weight (viscosity 200.000–800.000 cps) with a degree of deacetylation of 85% was purchased from Sigma–Aldrich (St. Louis, MO), acetic acid glacial (99.9% purity) and acetone (99.8% purity) were obtained from Carlo Erba Reagenti (Rodano, Mi – Italy) and carbon dioxide (99.5% purity) was bought from SON (Società Ossigeno Napoli – Italy). Water was distilled using a laboratory water distiller supplied by ISECO S.P.A. (St. Marcel, Ao – Italy). All materials were used as received.

2.2. Apparatus

Chitosan samples were prepared in a home-made laboratory plant that mainly consists of a 316 stainless steel cylindrical high-pressure vessel with an internal volume of 80 mL, equipped with a high pressure pump (Milton Roy – Milroyal B, France) used to deliver SC-CO_2. Pressure in the vessel was measured by a manometer (Mod. 0.25, OMET, Italy) and regulated by a micrometering valve (Mod. 1335G4Y, Hoke, SC, USA). Temperature was regulated using temperature controllers (mod. 305, Watlow, Italy). At the exit of the vessel, a rotameter (Mod. D6, ASA, Italy) was used to measure CO_2 flow rate.

2.3. Procedures

Chitosan solution was prepared by dissolving Chitosan (between 2 and 10% w/w) in water:acetic acid solutions with ratios of 99:1, 97:3 and 95:5; these solutions were stirred at 100 rpm and heated at 50 °C until they became homogenous. Then, they were poured in steel containers with a internal diameter of 2 cm and height of 1 cm and were phase-separated at a temperature of −20 °C for 24 h. Subsequently, the sample was put in a bath of acetone at −20 °C for 24 h to “substitute” water and, then, it was dried using SC-CO_2.

SC-CO_2 drying was performed according to the following procedure: the steel containers were loaded on a metallic support that was, then, put in the high pressure vessel. The vessel was closed and filled from the bottom with SC-CO_2. When the required pressure and temperature were obtained (200 bar and 35 °C), drying was performed with SC-CO_2 at a flow rate of about 1 kg/h, that corresponds to a residence time inside the vessel of about 4 min; the drying lasted 5 h. A depressurization time of 20 min was used to bring back the system at atmospheric pressure.

2.4. Characterizations

2.4.1. Scanning electron microscopy (SEM)

Chitosan scaffolds were cryofractured using liquid Nitrogen; then, the sample was sputter coated with Gold (Agar Auto Sputter Coater mod. 108 A, Stansted, UK) at 30 mA for 150 s and was analyzed by a Field Emission-Scanning Electron Microscope (Assing, mod. LEO 1525, Italy) to analyze cell and pore size and the overall scaffolds morphology. Sigma Scan Pro software (Jandel scientific version 5.0) and Origin Pro 8.5 (Microcal) software were used to measure the average value of the pores and to calculate pore diameter distributions.