Hybrid porous cryogels processed using pressurized CO_2 and freeze drying for life science applications

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ABSTRACT

This work is aimed to present a novel route towards bimodal porous scaffolds for tissue engineering and regenerative medicine (TERM) applications. The key idea of the route is to produce highly porous hydrogels by gelation of alginate/biopolymer mixture under pressurized carbon dioxide in presence of calcium cations. Hybrid cryogels with gelatine, gellan gum, carboxymethylcellulose and lignin were successfully prepared after freeze drying. Cryogels for TERM are of special interest due to large and highly interconnected pores that provide non-constrained mass transfer for cell growth and proliferation. However, to achieve desirable mechanical properties as well as high adsorption capacity, bimodal meso and macroporous materials are greatly desirable. Two freezing regimes were studied in order to convert hydrogels into cryogels, namely slow freezing at –80° C and rapid freezing in liquid nitrogen.

INTRODUCTION

Hydrogels are a three-dimensional, insoluble, cross-linked network of polymers that can imbibe large quantities of aqueous solutions. Hydrogels are excellent candidates for tissue engineering scaffolds because of their hydrophilic nature and mass transfer properties [1]. Factors affecting microstructure of the scaffolds such as pore size and interconnectivity of pores play an important role in determining cell proliferation, differentiation, and subsequent tissue formation. Macroporous hydrogels with interconnected pores not only provide sufficient surface area for cell attachment and proliferation but also allow enhanced mass transfer of oxygen, nutrients and waste removal [2]. Various methods have been used in the past to produce macroporous hydrogels such as gas foaming [3], fiber bonding [4], micro-emulsion formation [5], phase separation [6], freeze-drying [7] , and porogen leaching [8]. More recently, using high pressure carbon dioxide (CO_2) has been explored as a green process to synthesize three-dimensional (3D) hydrophilic structures with highly interconnected macroporous networks [9]. Gelation refers to the linking of macromolecular chains together which initially leads to progressively larger branched yet soluble polymers depending on the structure and conformation of the starting material. Gelation can take place either by physical

linking (physical gelation) or by chemical linking (chemical gelation). Hydrogels produced using high pressure CO_2 generally exhibit greater porosity and improved cross-linking, resulting in improved gel stiffness as well as an enhanced capacity to support cell and tissue infiltration [10]. Nonetheless, although these improvements are significant, it is important to note that residual cross-linking agents not recovered after processing or released during material degradation may result in adverse effects to biological systems. Cryogels are obtained after submit the hydrogels to the freezing process [11]. The structure of the same hydrated materials obtained by fast and slow freezing in final have differences, these is based on the common view that fast or slow freezing of water results in the formation of smaller (less ordered) or larger (more ordered) ice crystallites, respectively. Therefore, the structure of the frozen hydrated materials depends both on the amount of water and freezing rate. The slower the freezing rate, the larger the ice crystallites formed, which can destroy pore walls, cellular membranes, whole cells, etc. These effects are due to a larger volume of ice crystallites than related water droplets [12].

MATERIALS AND METHODS

Materials

Gelzan CM (gellan gum), alginic acid sodium salt, gelatin and carboxymethylcellulose were purchased from Sigma-Aldrich (Germany). Calcium carbonate was supplied by Magnesia GmbH (Germany), lignin was produced by enzyme hydrolysis at the TUHH. Ethanol for solvent exchange was obtained from H. Möller GmbH & Co.KG (Germany). Carbon dioxide was supplied by AGA Gas GmbH (Germany). All other chemical reagents were ACS reagent grade and were used as received.

Alginate-based cryogels preparation

Hybrid hydrogels (1.5 wt% alginate/1.5 wt% biopolymer) with gelatin (Glt), gellan gum (GG), carboxymethylcellulose (CMC) and lignin (Lig) were prepared. Polymers were dissolved in distilled water, and stirred for 1 hour. The gelation were performed using carbon dioxide (CO_2) in a pressure vessel with 30-60 bar at 20 – 60 ºC. Two freezing regimes were studied in order to convert alginate-based hydrogels into cryogels, namely slow freezing at –80° C and rapid freezing in liquid nitrogen. Finally frozen materials were freeze dried.

Characterization of alginate-based cryogels

Hybrid cryogels prepared under high pressure CO_2 were analysed using different analytical techniques. Textural properties of the scaffolds were analyzed by SEM and micro-computed tomography. The mechanical properties of the cryogels were characterized in compression mode (wet and dry state). The fluid uptake capability and the weight loss were measured for a period up to 14 days by the immersion of the samples in 10 ml of culture medium at 37ºC and 60 rpm. Finally, the cytotoxicity of the cryogels was assessed by MTS assay in accordance with ISO/EN 10993, using an immortalized mouse lung fibroblasts cell line (L929) purchased from the European Collection of Cell Cultures. The effect of the

leachable released from the cryogels on the cellular metabolism was evaluated for after 72h of culture.

RESULTS

In this work, hybrid alginate-based cryogels obtianed after gelation under pressurized carbon dioxide were tested for tissue engineering and regenerative medicine (TERM) applications. The cross-link of hydrogels occurs due to the decrease of pH, promoted by the dissolution of carbon dioxide in the aqueous phase. Gelation is, hereafter, favoured within an acid environment, which is attained a pressure increase [9]. In our case, the gelation is further promoted by the presence of calcium carbonate in the polymer solution, which allows the cross-link of alginate-based hydrogels inside the pressure vessel.

In order to convert hydrogels into cryogels, two freezing regimes were studied, the slow freezing at –80° C and rapid freezing in liquid nitrogen (**Fig. 1**). Cryogels, with a 6 mm of diameter and 10 mm of height were prepared and characterized in terms of surface morphology by SEM.

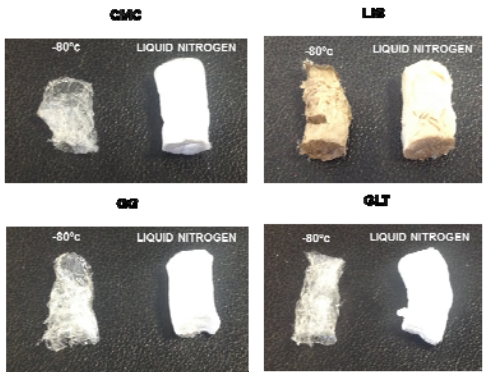

Figure 1. *Cryogels dried by two freezing regimes, the slow freezing (-80° C) and rapid freezing (liquid nitrogen).*

In terms of macro morphology the differences of two freezing regimes are visible in **Fig. 1**. The cryogels freezing by –80º C (slow freezing) have a more irregular shape and less compact when comparing with the cryogels freezing in liquid nitrogen (rapid freezing). The results of SEM images indicate that slow freezing (–80º C) lead to purely macroporous materials, whereas rapid freezing in liquid nitrogen resulted in both meso and macroporous structures (**Fig. 2**).

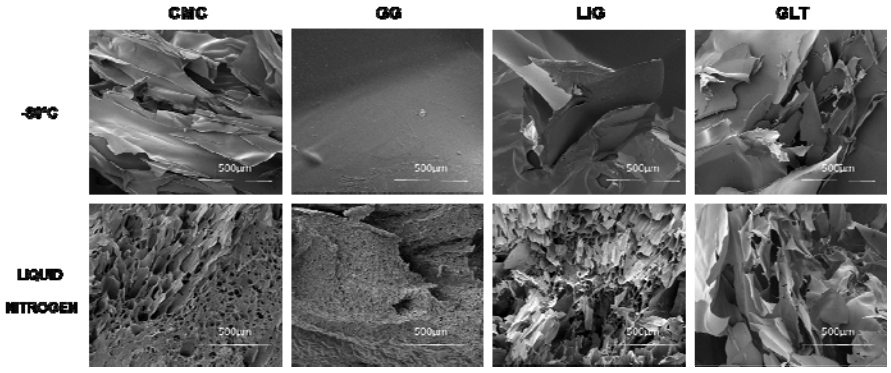

Figure 2. *SEM images of cryogels dried by two freezing regimes, the slow freezing (–80° C) and rapid freezing (liquid nitrogen).*

Due the higher porosity observed in the SEM analysis of hybrid cryogels dried by rapid freezing (liquid nitrogen) these were select for next studies. Morphological characteristics such as porosity, mean pore size and interconnectivity are determining factors that define the applicability of a matrix for TERM applications. Generally, a hydrogel with high porosity favors cellular growth, as a greater area is available for cell adhesion and migration; this, in turn, facilitates the proliferation of the cells. Micro-computed tomography (μ-CT) was used to evaluate the porosity and pore size of the alginate-based cryogels prepared under high pressure CO_2. The micro-CT analysis showed higher values for alginate/GG cryogel. The mean pore size of hybrid cryogels was approximately 190 μm and the porosity of 52 %. The lower values were obtained in the case of alginate/GLT with mean pore size 90 μm and porosity of 27 %. In **Table 1** the results obtained by μ-CT for all hybrid cryogels are presented. The values of porosity, mean pore size and interconnectivity suggest the possibility of cells to colonize and migrate through the hybrid cryogels prepared under high pressure CO_2.

Table 1 - *Results obtained by micro-CT analysis for hybrid cryogels prepared under high pressure CO_2.*

Sample	Porosity (%)	Mean pore size (μm)	Interconnectivity (%)
CMC	39.5 ±0.3	126±13	50.9±0.7
GG	66.8 ±0.7	198±7	51.1±3.6
GLT	37.9±1.2	90±6	27.2±1.4
LIG	40.3±3.1	127±15	27.1±9.3

To ascertain the mechanical performance of alginate-based cryogels prepared under high pressure CO_2, mechanical compression experiments were performed. The compressive Young´s modulus show that the values are approximately 1 MPa in dry state for all formulations. The wet and dry state of compressive Young´s modulus were compared and it was observed a decrease of values around two orders of magnitude after hydrated the cryogels in culture medium. The fluid uptake analysis demonstrate a swelling ratio around 28% in all formulations. Likewise the weight loss after 7 days for all formulations was around 25%.

The cytotoxicity of the cryogels was assessed by MTS assay in accordance with ISO/EN 10993, using an immortalized mouse lung fibroblasts cell line (L929) and the

cryogels made by gelatin, gellan gum, carboxymethylcellulose and lignin do not present cytotoxicity and have hereafter the potential to be used for tissue engineering and regenerative medicine.

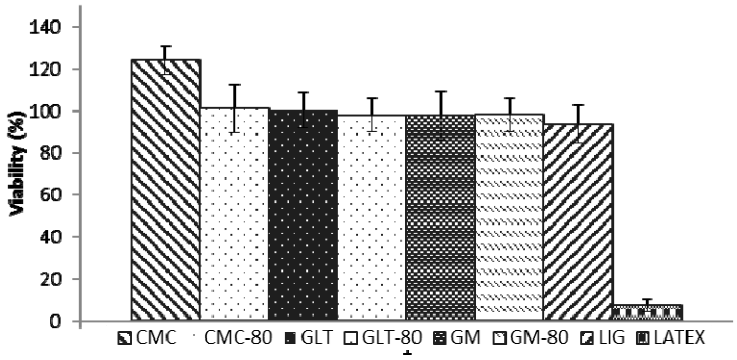

Figure 3. *Cell viability measured after 72 hours of cryogels.*

CONCLUSION

Hybrid alginate cryogels blended with gelatin, gellan gum, carboxymethylcellulose and lignin were successfully prepared by gelation under pressure. Rapid freezing with subsequent freeze-drying leads to high porosity both in meso and macro range. The results obtained suggest that developed cryogels have, hereafter, the potential to be used for tissue engineering and regenerative medicine.

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