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In situ Raman-analysis of supercritical carbon dioxide drying applied to acellular esophageal matrix

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ABSTRACT

Supercritical (SC) carbon dioxide (CO_2) drying has shown potential interest on the production of dry decellularized extracellular matrices for tissue engineering. This work explores the feasibility of Raman spectroscopy as *in situ* analysis for the SC-CO_2 drying monitoring of a decellularized esophagus. Esophagus tissue was dried within an optical accessible high pressure CO_2 dryer while the Raman signals of the wet tissue were excited at 785 nm.

Two different experimental procedures were explored: i) single step process (SC-CO_2 drying alone) and ii) two steps process (water exchange by ethanol followed by SC-CO_2 drying). The quantification of the removal of water or ethanol from the tissue during the drying was obtained evaluating the variation of the Raman signal intensity ratio during time. The measurement of water or ethanol removal curves during the drying process can be easily/successfully exploited for the *in situ* monitoring of the drying process of a general matrix/tissue.

1. Introduction

Esophagus diseases may require surgical treatments and substitution with high risk of morbidity and post surgical complications [1]. Tissue engineering of esophagus is emerging for the development of novel engineered substitutes using several synthetic and natural scaffolds [2]. The use of decellularized matrices is promising for the organ/tissue replacement with evidence of satisfactory clinical experience [3,4]. These natural acellular matrices could represent a valid therapeutic solution for the replacement of damaged organs, overcoming rejection and immunosuppression complication. These scaffolds retain the architecture and ultrastructure of the original (o native) tissue, which is essential to promote cellular orientation and proliferation. Furthermore, they may act by mediating immunological response promoting cellular survival after implantation [5]. However some limitations, like long term preservation before implantation or residual toxicity, should be solved since they limit the clinical application of such technology. Our group has recently investigated the effect of SC-CO_2 drying for the successful development of dry natural-based engineered tissues useful as alternative esophageal substitution [6]. We demonstrated that SC-CO_2 drying alone or in a combination with a pre-dehydration with ethanol (EtOH) represents a promising innovative technique for the preparation of novel a-toxic organ/tissue substitution, in absolute dry conditions and with a suitable structure for cell re-population.

Despite the potential of the SC-CO_2 drying technology, additional analysis of the process should be added in order to optimize the operational process conditions, thus making the procedure standardized. For example SC-CO_2 drying treatment is dependent on the thickness/weight of the sample and these parameters can influence the SC-CO_2 drying time needed to achieve a satisfactory dehydration. Additionally, due to the natural source of the scaffold, the decellularized tissues are affected by high variability which influences the process conditions to be used, in particular, the operative pressure or the treatment time needed to achieve a satisfactory drying process. This intrinsic variability is not easy to be controlled and almost impossible to estimate before each treatment. Moreover, in a clinical scenario of application, high quality standard must be guaranteed and the process should be continuously monitored to ensure the absence of toxic components, such as residuals of the enzymatic treatment and solvents used.

Monitoring the water removal is considered the main indicator of the achievement of drying. Usually the water removal analysis is

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achieved measuring the loss of weight between the beginning and the end of the drying. However such a sampling method is not always feasible for natural based process where high process variability can highly influence the achievement of the drying condition. Using off line measurements as indicator of drying can be inconvenient since it can lead to waste of product and therefore to an increase of entire cost of the process. The online analysis of product parameters, such as water removal, inside a high pressure vessel is very challenging.

In this regards, Raman spectroscopy has been demonstrated to be a promising and one of the most informative non-invasive probes to investigate *in situ* the properties of different materials under extreme conditions of high pressure [7]. Raman spectroscopy was used as *in situ* measurement tool at elevated pressures and in the context with SC-CO_2 in various fields, such as for the analysis of the supercritical drying of silica gels [8], for the analysis of the supercritical antisolvent process [9] or for the analysis of the dissolution kinetics of compressed CO_2 into yeasts solutions [10]. Also *in situ* absorption based techniques have been successfully applied for the analysis of drying processes, applied in thin surface layers of polymers and other tissues or in the fluids surrounding the tissue [11].

In the present work *in situ* Raman technique was exploited, for the first time, to monitor the SC-CO_2 drying process of natural decellularized esophagus by using a small optically accessible high pressure lab-scale vessel.

Two different experimental procedures were explored: a single step SC-CO_2 drying at 35 °C and 10 MPa up to 300 min, and a combined double step in which a pre-hydration with ethanol (EtOH) for 80 min is followed by SC-CO_2 drying at 35 °C and 10 MPa up to 120 min [6]. The Raman spectra were evaluated and an *ad-hoc* strategy for monitoring both, the kinetics of water and EtOH removal during the process is described.

2. Methods

2.1. Sample preparation

The organ harvest and the acellular matrix generation procedures were reported in our previous work [6]. Decellularized esophagi were processed within 1 month after the detergent enzymatic treatment (DET). All the esophagi were stored at 4 °C in phosphate buffer solution (PBS, Life Technology) containing 1% of Penicillin-Streptomycin (Life Technologies) before their drying. The decellularized matrices were obtained from the cervical portion and the samples were prepared by cutting the esophagi in cylinders of 5 cm length using sterile cutters and tweezers under a biological safety cabinet. In Fig. 1 a photograph of a decellularized esophagus cylinder used for the experiments is shown.

2.2. Dehydration and drying processes

The supercritical drying process was performed in a high pressure sapphire visualization cell (Separex S.A.S., France) as previously reported [6] using a semi-continuous mode (CO_2 flow rate 23 mL/min). SC-CO_2 drying treatments were performed at 35 °C and 10 MPa for a treatment time up to 300 min when only SC-CO_2 was applied, while of 80 min when the samples were firstly dehydrated with a ethanol solution. The dehydration (or solvent exchange) was performed in a glass pirex container dipping the samples in series of ethanol/water solutions with various ethanol contents (volume fraction 50%, 75%, 95%, and 99.8%) for 20 min at each step.

Samples were weighted before and after each drying treatment using a microbalance (Sartorius- ED224S). The weight reduction was expressed in% ($1-m_{end}/m_{start}$) where m_{end} and m_{start} were respectively the mass of the sample after and before the drying treatment. For comparison, we also carried out thermal air drying treatment in an oven (fratelli Galli-Italy) at 80 °C overnight.

2.3. Raman measurement

The experimental set up is shown in Fig. 1 when the decellularized esophagus cylinder was inserted inside the high pressure vessel. Raman measurements were made with a diode laser (Toptica DLpro) operated at 785 nm. The laser output power was less than 500 mW, approximately one fifth of the laser power reaches the measurement object, which we know from comparing the signal strength detectable from esophagus positioned inside and outside the drying chamber. The remaining four fifth of the laser power is deflected away when the laser beam passes the cylindrical double tube sapphire jacket of the drying-chamber. The laser did not degenerate the sample at this setting. Also the Raman signals originating from the esophagus inside the drying vessel were attenuated significantly – similar to the excitation laser – as they also had to pass the cylindrical sapphire window.

The excitation light from the laser source was guided to the Raman sensor head through a glass fiber and is then firstly collimated by a convex lens, then guided *via* a dichroic mirror towards the sample and focused *via* another convex lens into the tissue. The Raman signals scattered from the tissue (center of probe volume) are imaged onto the glass fiber detection bundle using the same lens which already focused the excitation laser and a second convex lens to focus the signals on the

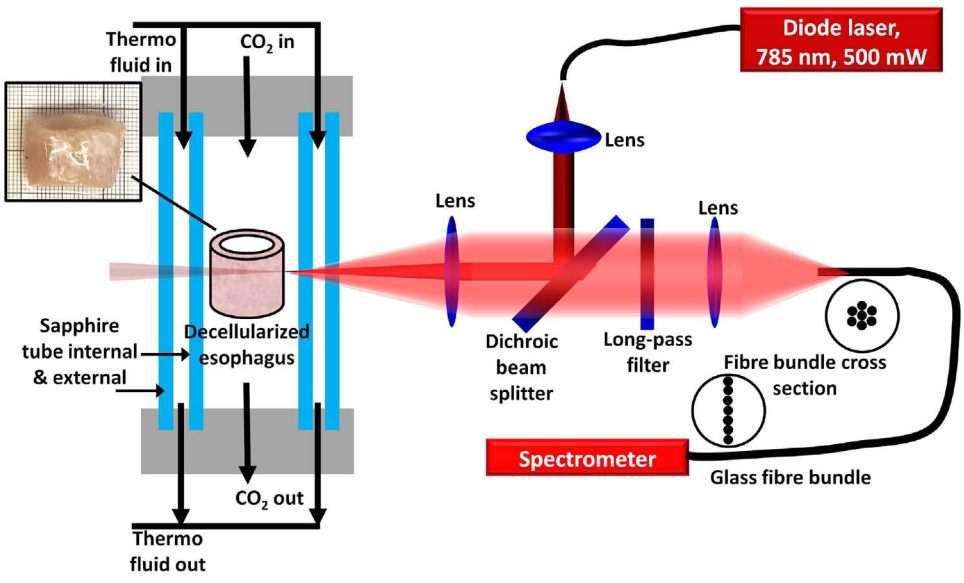

Fig. 1. Schematic representation of the experimental setup and picture of the decellularized esophagus cylinder.