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Supercritical carbon dioxide extracted extracellular matrix material from adipose tissue

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ABSTRACT

Adipose tissue is a rich source of extracellular matrix (ECM) material that can be isolated by delipidating and decellularizing the tissue. However, the current delipidation and decellularization methods either involve tedious and lengthy processes or require toxic chemicals, which may result in the elimination of vital proteins and growth factors found in the ECM. Hence, an alternative delipidation and decellularization method for adipose tissue was developed using supercritical carbon dioxide (SC-CO_2) that eliminates the need of any harsh chemicals and also reduces the amount of processing time required. The resultant SC-CO_2-treated ECM material showed an absence of nuclear content but the preservation of key proteins such as collagen Type I, collagen Type III, collagen Type IV, elastin, fibronectin and laminin. In addition, other biological factors such as glycosaminoglycans (GAGs) and growth factors such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) were also retained. Subsequently, the resulting SC-CO_2-treated ECM material was used as a bioactive coating on tissue culture plastic (TCP). Four different cell types including adipose tissue-derived mesenchymal stem cells (ASCs), human umbilical vein endothelial cells (HUVECs), immortalized human keratinocyte (HaCaT) cells and human monocytic leukemia cells (THP-1) were used in this study to show that the SC-CO_2-treated ECM coating can be potentially used for various biomedical applications. The SC-CO_2-treated ECM material showed improved cell-material interactions for all cell types tested. In addition, *in vitro* scratch wound assay using HaCaT cells showed that the presence of SC-CO_2-treated ECM material enhanced keratinocyte migration whilst the *in vitro* cellular studies using THP-1-derived macrophages showed that the SC-CO_2-treated ECM material did not evoke pro-inflammatory responses from the THP-1-derived macrophages. Overall, this study shows the efficacy of SC-CO_2 method for delipidation and decellularization of adipose tissue whilst retaining its ECM and its subsequent utilization as a bioactive surface coating material for soft tissue engineering, angiogenesis and wound healing applications.

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1. Introduction

The properties of a biomaterial surface have been shown to play instructive roles in the regulation of cell-material interactions and for controlling cellular functions such as adhesion, migration, proliferation and differentiation [1]. However, most biomaterials may possess the desired bulk properties but not the surface properties that meet the required bio-application needs. Hence, surface modification of the biomaterial with a bioactive component is an effective method to improve surface biofunctionality whilst maintaining the bulk property of the material [2].

The extracellular matrix (ECM) is an example of a bioactive material that is made up of a mixture of complex structural and functional proteins, proteoglycans and biological factors that influences cell adhesion and growth, directs cellular fate, and modulates host tissue responses [3–6]. Consequently, the ECM can potentially be used as a surface coating material to confer bioactivity or improve cell-material interactions of other materials. Presently, various individual components of ECM, such as collagen [7], laminin [8] and fibronectin [9], have been used as

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a surface coating material to improve cell-material interactions. Previously our group has shown the promising utility of decellularized lipoaspirate material for conferring bioactivity to otherwise bioinert polycaprolactone (PCL) substrates, as well as to protein-based microcarriers [10,11].

Currently, adipose tissue, in the form of lipoaspirate material derived from plastic surgery and reconstructive procedures, is generally treated as clinical waste. Nevertheless, adipose tissue has also been found to contain a large amount of ECM material [12]. Hence, SC-CO_2-treated ECM, as a material, can potentially be used as a bioactive coating material for enhancing cell-material interactions. However, the high lipid content of adipose tissue poses unique challenges in conventional delipidation and decellularization procedures. In addition, current delipidation and decellularization methods either require tedious and lengthy processes using enzymatic digestion or involve the use of harsh chemicals such as sodium dodecyl sulfate (SDS) [4,12]. However, it is relatively difficult to ensure the complete removal of all the toxic chemicals prior to usage. Furthermore, the components of the ECM are often damaged as the result of these toxic and harsh chemicals. As such, an alternative method for delipidating and decellularizing the lipoaspirate tissue that eliminates the need for harsh chemicals whilst requiring a shorter processing time, is highly desirable.

Since the end of the 1970s, supercritical fluids have been used to isolate natural products such as the extraction of fish oil, microalgae lipids or bioactive compounds from marine resources [13–15]. In fact, this method has been well recognized as an environmentally friendly processing technique that is regarded safe by the Food and Drug Administration (FDA) [13,16,17]. In the recent years, supercritical fluids have also been used for the removal of cells from porcine aorta to produce acellular artificial tissue or as a scaffold fabrication technique to produce porous scaffolds and grafts for tissue engineering applications [18–22]. However, limited studies have been carried out using supercritical fluids on surgical waste materials such as lipoaspirate tissue, which is a rich source of ECM material. Hence in this study, a green processing method using supercritical carbon dioxide (SC-CO_2) was developed for the delipidation and decellularization of the lipoaspirate tissue to obtain SC-CO_2-treated ECM material. The SC-CO_2-treated ECM material was then characterized to reveal the various proteins and growth factors preserved within the SC-CO_2-treated ECM material. Subsequently, the entire SC-CO_2-treated ECM material was used as a bioactive coating to improve cell-material interactions. Overall, four different types of cells that are commonly used in soft tissue engineering research (*i.e.* adipose tissue-derived mesenchymal stem cells (ASCs), human umbilical vein endothelial cells (HUVECs), immortalized human keratinocyte (HaCaT) cells and human monocytic leukemia cells (THP-1) were cultured on the surfaces of tissue culture plastic coated with SC-CO_2-treated ECM material (TCP-ECM) and the outcome of SC-CO_2-treated ECM enrichment on cell-material interactions was investigated.

2. Materials and methods

2.1. Materials

Freshly isolated lipoaspirate tissue was obtained from patients at Tan Tock Seng Hospital (TTSH), Singapore following procedures established by the National Healthcare Group Domain Specific Review Board (NHG DSRB 2012/00071) [10,11]. The lipoaspirate tissue was transported to the laboratory within 4 h of harvesting. All the chemicals used were ordered from Sigma-Aldrich, USA unless stated otherwise.

2.2. SC-CO_2 treatment of lipoaspirate tissue

The schematic diagram of the overall set-up used to decellularize the lipoaspirate tissue is shown in Fig. 1. Lipoaspirate tissue was first rinsed with distilled (DI) water in order to remove the blood components completely. Next, the lipoaspirate tissue was rinsed twice in ethanol

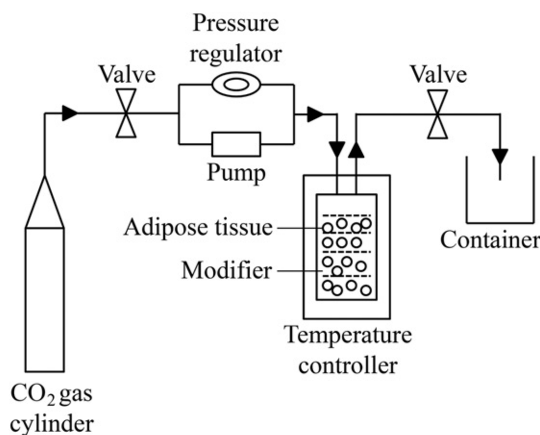

Fig. 1. Schematic view of the overall set-up for the SC-CO_2 treatment used for the delipidation and decellularization of lipoaspirate tissue. (Arrows indicate the process flow direction).

(Merck Millipore, USA) for 10 min. The lipoaspirate tissue was then loaded into the reaction vessel (Supercritical Fluid Technologies Inc., USA) with ethanol as modifier. Liquid carbon dioxide (CO_2) was then flowed into the reaction vessel in the direction of the pointed arrows in Fig. 1. CO_2 was first compressed by a pump and then passed through the regulator until the desired pressure (180 bar) was achieved. The temperature of the reaction vessel was maintained at 37 °C for 3 h. Finally, the SC-CO_2-treated ECM material was collected and stored at 4 °C until required.

2.3. Characterization of SC-CO_2-treated ECM material

2.3.1. Confirmation of delipidation and decellularization

The morphology of the lipoaspirate tissue and SC-CO_2-treated ECM material was investigated using the scanning electron microscope (SEM; JEOL Co., Japan) following established protocols [23]. Briefly, the samples were first prepared by fixing them in 2.5% glutaraldehyde solution at 4 °C for 24 h. The samples were subsequently rinsed with DI water, dehydrated in a graded ethanol series followed by 100% hexamethyldisilazane (HMDS) and air dried. The samples were then sputter-coated with gold using SPI Module Sputter Coater (SPI Supplies Inc., USA) and viewed at an acceleration voltage of 5 kV at a magnification of $\times 200$.

Oil Red O staining was performed to confirm the successful delipidation of the lipoaspirate tissue, whilst hematoxylin and eosin (H&E) staining (Merck Millipore, Germany) was performed to confirm the successful decellularization of the lipoaspirate tissue according to previously established protocols [11,23,24]. Fresh lipoaspirate tissue was used as a control. The samples for Oil Red O staining were cryosectioned prior to staining. Briefly, the samples were fixed in 4% paraformaldehyde solution for 24 h followed by rinsing with 1× phosphate buffered saline (PBS) solution (Life Technologies™, USA). The samples were then embedded in tissue freezing medium (Leica Biosystems, Germany) and kept frozen at −20 °C. Subsequently, the samples were cryosectioned into 60 μm thick sections using a microtome machine (Leica Biosystems, Germany). Next, the sections were stained with Oil Red O working solution by dissolving 1.8 mg of Oil Red O powder in 1 mL of 60% isopropanol solution (Merck Millipore, Germany) to detect the presence of lipids. On the other hand, samples for H&E staining were paraffin embedded and sectioned prior to staining. Briefly, the samples were fixed in 4% paraformaldehyde solution for 24 h followed by rinsing with 1× PBS solution. The paraformaldehyde fixed samples were then subjected to gradient ethanol dehydration (50, 60, 70, 80, 90, 100% v/v in water) followed by immersing the samples in a second 100% ethanol solution overnight. Subsequently, the samples were treated with 1:1 of ethanol:xylene and pure xylene

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