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Fabrication of nanofibrous microcarriers mimicking extracellular matrix for functional microtissue formation and cartilage regeneration

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ABSTRACT

Cartilage has rather limited capacities for self-repair and regeneration. To repair complexly shaped cartilage tissue defects, we propose the application of microtissues fabricated from bone marrow-derived mesenchymal stem cells (BMSCs) cultured in natural bionic nanofibrous microcarriers (NF-MCs). The NF-MCs were structurally and functionally designed to mimic natural extracellular matrix (ECM) by cross-linking dialdehyde bacterial cellulose (DBC) with DL-allo-hydroxylysine (DHYL) and complexing chitosan (CS) with DHYL through electrostatic interactions. The orthogonal design allows for fine tuning of fiber diameter, pore size, porosity, mechanical properties, and biodegradation rate of the NF-MC. BMSCs cultured in NF-MCs showed improved proliferation compared with those cultured in chitosan microcarriers (CS-MCs). After three-week culture under microgravity conditions, functional cartilage microtissues were generated. When implanted into a knee articular cartilage defect in mice, the microtissue showed superior *in vivo* cartilage repair as characterized by cell tracking, histology, micro CT image, and gait analysis. Versatile in natural biopolymer design and biomimetic in nanofibrous component embedded in macroporous microcarriers, these injectable NC-MCs demonstrate to be effective carriers for cell proliferation and differentiation. Furthermore, the functional microtissues also show their prospect in repair of cartilage tissue, and suggest their potential for other tissues in general.

1. Introduction

Cartilage has rather limited capacities for self-repair and regeneration owing to its unique avascular microstructure [1]. Recently various treatment methods for cartilage injury have been explored to help repair damaged cartilage [2]. Among them, tissue engineering (TE) which demonstrates great clinical potential for regeneration of hyaline-like cartilage tissue and treatment of chondral lesions is considered to be a promising alternative strategy for cartilage repair [3]. Conventional TE approaches are usually based on the combination of bulk three-dimensional (3D) scaffolds with cell sources and growth factors to construct artificial tissues *in vitro* prior to implantation [4]. However, these approaches often present limitations in clinics when used to fill and repair irregularly shaped defects in cartilage. To this end, microtissue engineering, which utilizing cell-loaded microcarriers as building blocks for implantable/injectable treatment, is designed in order to achieve an accurate fit with defect sites. Microtissue is constructed using microcarriers and cells, and then transplanted into a custom bioreactor to form a tissue or organ [5,6]. The greatest benefit of microtissue engineering is that the functional microtissue with high activity can be easily delivered to the sites through implantation, thereby eliminating the digestion of cells before transfer in monolayer culture from flasks [7].

Since most seeded cells are adherent, good adhesion on the microcarrier for growth and maintenance of proliferation and function *in vitro* for constructing microtissue is the key technology, especially for cartilage engineering [8]. In other words, the microcarriers play a vital role in the formation of functional microtissues. Microcarriers offer a series of desirable advantages, including maintaining the cell differentiated phenotype and inducing cell

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proliferation. Microcarriers is often processed to produce rough, multiporous and hollow structures for cell attachment and proliferation. Traditional microcarriers can be divided into synthetic microcarriers and natural microcarriers. Synthetic microcarriers include polystyrene microcarriers, polyacrylamide microcarriers and glucose microcarriers. But almost all of them lack cell recognition sites and affect cell growth [9]. Therefore, researchers are now using natural polymers because they are easily obtained and biocompatible [10,11]. Gelatin, collagen, alginate as well as chitin and its derivative chitosan have already been used as substrates to fabricate microcarriers for cartilage repair [12–14]. However, there are still certain shortcomings with natural microcarriers. A potential disadvantage is possible diffusion limitations for nutrients and oxygen that might result in necrotic foci deep within the microcarrier [15,16]. Another crucial issue is the cell source. Most of the recent studies have been focused on using chondrocytes loaded microcarriers for cartilage regeneration [14,17,18], but drawbacks such as donor site morbidity, inadequate chondrocyte supply, chondrocyte dedifferentiation in monolayer culture, and periosteal hypertrophy were reported [19,20]. In addition, present natural microcarriers also exhibit limitations when used *in vivo*, such as irregular specific surface, low mechanical strength and fast degradation rate. Also, despite the popular use of collagen microcarriers due to their biological functions, their immunogenicity has limited their further applications. Therefore, to improve the properties of the microcarriers in order to obtain better cell adhesion and proliferation thereby forming favorable cartilage microtissue, the cartilage microcarriers should be specially treated [21].

Natural ECM which composed of an interlocking mesh of fibrous proteins and glycosaminoglycans (GAGs) [22], could promote cell growth activity and affect the differentiation [23,24]. Many researchers have already used ECM components to construct microcarriers for tissue engineering [23–26]. However, natural ECM is limited in source, rapid *in vivo* degradation and unable to provide mechanical support for tissue defects repair, thereby restricting its clinical application. Besides, immunogenicity is also an important issue which should not be ignored. In recent years, many researches have provided evidence that the ECM–mimicking nanofibrous architecture advantageously enhances cell-material interactions; channels/pores at multiple scales (between spheres, within spheres and between nanofibers) promote cell migration [27], proliferation and mass transport conditions, facilitating tissue regeneration and integration with the host [17,28]. Hence, in order to promote cell growth, proliferation and eventually obtain functional microtissues, we expect to use simple methods to design and prepare novel microcarriers with the similar complex functions as the cartilage ECM.

As an artificial ECM, a good scaffolding material should mimic the advantageous features of the natural ECM. An ideal tissue repair material should be able to mimic the mechanical and biological functionality of the ECM. Therefore, to construct ECM-mimicking microcarriers, several materials are involved. Oxidized bacterial cellulose (DBC), which is a nanofibrous cellulosic material, provides the natural nanofibrous structure resemble to the ECM [29,30], and has sufficient mechanical strength to prevent the microstrand from collapse due to the contraction forces by the surrounding cells [31]. Hydroxylysine is an important component of collagen, which is especially abundant in cartilage type II collagen. In previous studies, it was reported that hydroxylysine offers several excellent properties, including non-toxicity, suitable biodegradability, good cyocompatibility and low immunogenicity [32,33]. Especially, when cultured with cells, hydroxylysine promotes cell differentiation and facilitates the chondrogenesis [34,35]. Chitosan is a linear polysaccharide with good biocompatibility which shares some structural characteristics with GAGs and hyaluronic acid present in articular cartilage [31]. Microcarrier fabricated by chitosan has been widely used in tissue engineering, however, poor mechanical properties as well as low chondrogenesis potential restrict its further applications.

In this work, we designed novel ECM-mimicking microcarriers using natural nanofibers to mimic the collagen microfibers, and hydroxylysine and chitosan to create the similar cell growth microenvironment to cartilage ECM. The influence of various factors, including chitosan concentration, porosity, pore size as well as the mechanical and degradation properties on the nanofibrous microcarriers were investigated. We used bone marrow-derived mesenchymal stem cells (BMSCs) as cell source because they showed better cell arrangement, subchondral bone regeneration and integration than mature chondrocytes in various cartilage repair animal models [36]. Cell viability and cytocompatibility was then examined to determine whether the microcarriers could be effective carriers for cell growth and proliferation *in vitro*. Furthermore, we used these microcarriers to construct functional microtissues under microgravity culture conditions. Finally, the microcarriers as well as microtissue was directly implanted into knee articular cartilage defect in Sprague-Dawley rats respectively, to evaluate the cartilage regeneration.

2. Materials and methods

2.1. Preparation and characterization of C2, 3-dialdehyde bacterial cellulose (DBC)

BC (0.1 g) was oxidized by sodium periodate ($NaIO_4$) solution (0.2 mol/L, 100 mL, pH = 3.0) at 45 °C for various reaction times (1 h, 2 h, 3 h, 4 h, 5 h) under dark condition. After the remaining periodate was decomposed with excess ethylene glycol at 45 °C for 0.5 h under dark condition, the DBC was thoroughly washed by deionized water for three times and lyophilized for 24 h. The recovery rate (R, %) was calculated as following Eq. (1):

$$R(\%) = \frac{W_{DBC}}{W_{BC}} \times 100\% \quad (1)$$

where W_{BC} (g) is the dry weight of sample before oxidation, W_{DBC} (g) is the dry weight of the same sample after oxidation.

The aldehyde content of DBC was evaluated by Schiff base reaction with hydroxylamine [37]. Briefly, DBC (0.1 g) was suspended in 50 mL hydroxylamine hydrochloride – anhydrous methanol solution (0.3 mol/L), followed by addition of eight drops of thymol blue solution as indicator. The mixture was stirred in an incubator at the temperature of 37 °C for 24 h. The hydrochloric acid (HCl) produced by reaction between aldehyde and hydroxylamine was titrated with standard sodium NaOH – anhydrous methanol solution (0.01 mol/L). The endpoint was determined by macroscopic color change of the solution from red to yellow. Here, the consumption of NaOH solution was recorded as VE in ml. Moreover, the results were corrected by the blank group (the mixture of 50 mL hydroxylamine hydrochloride – anhydrous methanol solution (0.3 mol/L) with eight drops of thymol blue solution), and its consumption of alkali solution in mL was recorded as VB. Hence, the conversion of aldehyde to oxime was analyzed by the consumption of NaOH. The aldehyde content (AD, mmol/g) in DBC was calculated as followed:

$$AD(mmol/g) = \frac{0.01 \times (V_E - V_B)}{W} \quad (2)$$

The degree of oxidation (DO, %) was further calculated using the following Eq. (3):